Jitendra Kumar · Aditya Pratap
Shiv Kumar *Editors*

Phenomics in Crop Plants: Trends, Options and Limitations

Springer

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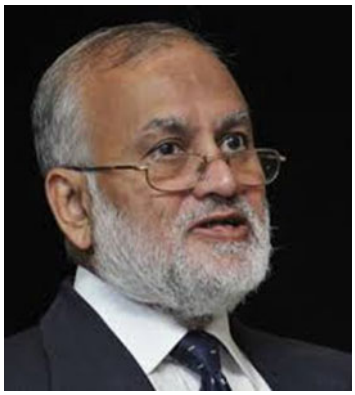
Foreword



Growing world population is expected to cause a "perfect storm" of food, energy and water shortages by 2030 as demand for food and energy will jump by 50 % and for fresh water by 30 %, as the population tops at 8.3 billion. The overarching challenge before the policy makers and agricultural scientists is how to ensure food and nutrition security for an ever-increasing population from limited and fast depleting resources under climate change scenario, especially in countries like India where sizeable population is still suffering from the triple burden of malnutrition. To meet the future demand of agricultural production, we need to develop more productive and nutritious varieties of agricultural crops which incorporate both high intrinsic yield potential and resilience under climatic stresses. This requires discovery and deployment of superior but complex traits from the vast germplasm resources being held in various gene banks to agronomically superior varieties efficiently and precisely.

Traits of breeders' interest such as grain yield, plant growth and resistance to biotic and abiotic stresses are complex as these are controlled by many genes of minor effects and highly influenced by environmental factors and their multi-dimensional interactions. In the past, plant breeders were successful in selecting desirable varieties empirically on the basis of visual observations, more so for qualitative traits; but empirical selection remains elusive and less effective for traits essential for meeting the current challenges such as underground, physiological and biochemical traits. Recent advances in genomics have created enormous genomic resources in several crop species which have the potential to increase harvestable yield

manifolds. However, available gene sequences and molecular markers could not be mainstreamed in crop improvement programs mainly due to the lack of precise phenotyping data. Therefore, it is imperative to phenotype the available germplasm precisely and efficiently in various crop species.

The current knowledge and voluminous information generated on phenotyping tools and techniques available in literature need to be consolidated so that researchers and scholars have access to such vast knowledge at one place. The present book, *Phenomics of Crop Plants: Trends, Options and Limitations*, which is a meticulously edited volume, is an attempt in this direction to bring together information on precision phenotyping under controlled versus natural environments, digital and image based phenotyping, phenomics of biotic and abiotic stresses and functional traits, and precision nutrient management. This book also covers experimental designs and biometrical approaches suitable for precision phenotyping of complex traits, and how phenomics can help to harness potentiality of genomics. Various chapters in this book have been contributed by renowned scientists whose research contributions are acknowledged globally. I am quite hopeful that the information contained in this book will boost research efforts of plant scientists to bring about a major breakthrough in agricultural production and will serve as a resource material for those who are involved in teaching and research in agricultural crops. I congratulate the editors Jitendra Kumar, Aditya Pratap and Shiv Kumar for bringing out this book timely on such an important and emerging aspect and hope that it would be widely read by scholars and researchers.

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Preface

It has been estimated that agricultural production must be doubled by 2050 in order to meet the predicted demand of growing world population. Achieving this goal poses a serious challenge to plant breeders as the current agricultural production growth rate of 1.3 % per annum is below the population growth rate. In the recent past, research advances have been made in the development of genomic tools and techniques which have the potential to increase the rate of genetic improvement. The whole genome studies have the potential to greatly facilitate genetic dissection of complex traits such as yield and stress tolerance by using technological advances in genotyping and sequencing. However, successful application of genomics towards the genetic improvement of crop plants depends upon our ability of precision phenotyping of these complex traits. Low cost and high-throughput genotyping has paved the way for the development of large mapping populations and diversity panels of thousands of recombinant inbred lines. These genetic resources require precise phenotyping. Marker-assisted recurrent selection (MARS) and genome-wide selection require phenotypic data, although conceptually selections are made on the basis of genetic information. A single phenotyping cycle is used to identify markers for subsequent selection through generations. In transgenic studies also, phenotyping is necessary for identification of promising events. Molecular breeding populations sometimes include up to 5,000 lines and their accurate characterization simultaneously is a challenging task. Also phenotyping of such complex traits are labor intensive, and many other interesting traits involved in biological processes are currently not suitable for genetic mapping due to the lack of approach to efficient and reliable measurement. The success in development of improved varieties relies on the ability to identify the best genetic variation for advancement. Because breeding is essentially a numbers game, more crosses and environments are required to identify superior variation with greater probability. Therefore, plant breeders want to phenotype a large number of lines rapidly and accurately to identify the best progeny. Advances in phenotyping are essential to capitalize on the developments in conventional, molecular, and transgenic breeding and ensure genetic improvement of crops for future food security.

In recent years, there has been increased interest in development of high-throughput phenotyping tools and techniques for screening of agronomic, physiological, and biochemical traits expressing especially under biotic and

abiotic stresses. These techniques have become much more advanced and have now entered the era of high-throughput field phenotyping. Several phenotyping platforms have been developed around the world, which are fully automated facilities in greenhouses or growth chambers with robotics, precise environmental control, and remote sensing techniques to assess plant growth and performance. Consequently, voluminous literature has been generated on different aspects of phenotyping which is scattered in numerous journals and books. However, no single publication is available to provide a comprehensive insight into this literature with a focus on phenomics of crop plants. This book, *Phenomics of Crop Plants: Trends, Options and Limitations*, is an attempt in this direction to bring together various high throughput, advanced phenotyping tools, techniques and platforms for directed genetic improvement in crop plants.

The present book comprises 19 chapters contributed by renowned scientists in their fields of expertise. The first chapter presents an overview on the recent developments in phenotyping. The second chapter deals with traits that require precise phenotyping. Chapter 3 discusses various issues related to phenotyping under controlled and natural environments while the subsequent three chapters (Chaps. 4, 5, and 6) deal with the imaging tools in phenotyping agronomic and physiological traits in crop plants. Chapters 7, 8, and 9 focus on phenotyping tools available for heat and drought related traits and soil problems. Chapter 10 deals with screening methods for diseases and possibility of using the recent developments in the field of phenomics. The subsequent three chapters (Chaps. 11, 12, and 13) discuss the advances in phenotyping of functional traits, role of florescence approaches for understanding the functional traits of photosynthesis and use of NMR in identification of subcellular structural and metabolic challenges. The next two chapters are on precision nutrient management and identification of nutritional and anti-nutritional factors of seeds (Chaps. 14 and 15). The subsequent two chapters (Chaps. 16 and 17) discuss the role of experimental designs for precision phenotyping and use of biometrical approaches in data analysis of the complex traits. As vast amount of genomic resources are now available in several crop plants, precision phenotyping can harness the potentiality of these genomic resources for accelerating the genetic improvement through mainstreaming them in the ongoing breeding programs. Therefore, the next two chapters (Chaps. 18 and 19) deal with how the available genomic resources can be utilized in a better way by using the available phenomics platforms worldwide for precise phenotyping of agronomic and physiological traits. Each chapter of this book has focused on the current trends, available options for phenotyping the target traits and limitations in their use for phenomics of crop plants.

The review of entire published work was neither possible in a single volume nor was the aim of this book. However, the contributors of individual chapters have provided exhaustive list of references on significant work done so far on different aspects of phenomics. Keeping in view the scope of the book, a little overlap in the subject is possible albeit all chapters have been dealt in depth by various experts. We are extremely grateful to all the authors

who despite being busy with their research and academics completed their chapters with a professional approach and great care.

We are highly indebted to Dr. S. Ayyappan, Secretary, Department of Agricultural Research and Education (DARE), Government of India, and Director General, Indian Council of Agricultural research (ICAR); and Dr. Mahmoud Solh, Director General, International Centre for Agricultural Research in the Dry Areas (ICARDA) for encouragement and inspiration in bringing out this publication. We are also thankful to Prof. Swapan Datta, Deputy Director General (Crop Science), ICAR; Dr. Maarten van Ginkel, Deputy Director General (Research), ICARDA; Dr. Michael Baum, Director of BIGM, ICARDA and Dr. B. B. Singh, Assistant Director General (Oilseed and Pulses), ICAR, for providing support and state-of-the-art facilities to carry out research on pulses. Dr. N. P. Singh, the present Director and Dr. N. Nadarajan, Ex-Director of IIPR, Dr. S. K. Chaturvedi, Head, Crop Improvement Division, IIPR, have been the source of encouragement for the present endeavor. Several people have rendered invaluable help in bringing this publication to life and they deserve our heartfelt appreciation and gratitude: Dr. Sanjeev Gupta, Project Coordinator, MULLaRP, IIPR, for technical comments and scientists of Crop Improvement Division, IIPR, for their valuable technical inputs during the course of editing the chapters; Mr. Ramesh Chandra, Senior Technical Assistant; Mr. Rohit Kant, Miss Nupur Malviya and Rakhi Tomar, Senior Research Fellows, for helping in compilation of references and voluminous correspondence, and Springer International for bringing the book through printing process with a thorough professional approach. Last but not least, our kids Neha, Gun and Puranjay and our better halves, Mrs. Renu Rani, Dr. Rakhi Gupta and Dr. Pankaj Rani Agrawal, deserve special thanks for their unstinting help, patience and emotional support during the course of this book.

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Dr. Jitendra Kumar, born in 1973, is presently working as a Senior Scientist in the Division of Crop Improvement at Indian Institute of Pulses Research, Kanpur. He has an excellent research career throughout. He secured Gold Medal during masters' programme and pursued his Ph.D. in Genetics and Plant Breeding from G. B. Pant University of Agriculture and Technology, Pantnagar, India. He was awarded CSIR-Research Associateship during 2003–2005 for postdoctoral studies at the Institute of Integrative Medicine, Jammu (India). He has more than 14 years of research experience in genetic improvement using both conventional and molecular marker-assisted breeding approaches on various crops including sunflower, medicinal and aromatic, cereal and pulse crops. He has done work on development of SSR markers, identification of QTLs for preharvest sprouting and high grain protein content and marker-assisted breeding in wheat for pyramiding the preharvest sprouting tolerance and high grain protein content and leaf rust resistance and developed a number of lines. During this period, he undertook study tours of several countries including Austria, Syria, Bangladesh, Nepal, Lebanon and Canada. His research interests include conventional and molecular breeding, QTL analysis and marker-assisted selection for crop improvement. He has more than 100 publications including research and review articles in reputed national and international journals, book chapters, meeting reports, popular articles, and bulletins. He has also co-edited three books including *Biology and Breeding of Food Legumes* published by CABI, Oxfordshire, *Alien Gene Transfer in Crop Plants: Innovations, Methods and Risk Assessment* and *Alien Gene Transfer in Crop Plants: Achievement and Impacts* both published by Springer, New York, USA. He has developed high-yielding varieties (IPL 316 and IPL 526) of lentil and several others are in the pipeline. His current priorities include involvement of molecular marker technology in conventional lentil breeding programme for making genetic improvement towards biotic and abiotic stresses.

Dr. Aditya Pratap, born on October 18, 1976, is currently working as a Senior Scientist (Plant Breeding) in the Crop Improvement Division, Indian Institute of Pulses Research, Kanpur. He obtained his Master's and Ph.D. degrees in Plant Breeding and Genetics from CSK Himachal Pradesh Agricultural University, Palampur, India, in 1999 and 2003. Holding a brilliant academic and service record, he has been associated with crop research since last 10 years. He has worked on genetic improvement of crop plants including wheat,

triticale, rapeseed-mustard, chickpea and *Vigna* species and has been instrumental in the development of haploidy breeding protocol in cereals through chromosome elimination technique. He has been associated with the development and release of five crop varieties including two in rapeseed-mustard (RSPT-2 and RSPR03), two in green gram (IPM 02-14 and IPM 02-3) and one in facultative winter wheat (DH 114). He has developed two extra early mungbean genotypes (IPM 205-7 and IPM 409-4 (48 days maturity)) besides being instrumental in establishing prebreeding garden of rapeseed-mustard at SKUAST-Jammu and of pulses at IIPR, Kanpur. Presently, he is working on genetic improvement of green gram (*Vigna radiata*) through distant hybridization aided by conventional and biotechnological tools. His research interests include distant hybridization, doubled haploidy breeding, plant tissue culture, and molecular breeding. To his credit, he has about 120 publications which include research papers published in high-impact journals as well as reviews/chapters for best international publishers including Springer, Academic Press and CRC. He has published four books entitled, *Haploidy Breeding in Triticale and Triticale X Wheat Hybrids: Comparison of Anther Culture and Chromosome Elimination Techniques* by Lambert Academic Publishing, Germany; *Biology and Breeding of Food Legumes* published by CABI, Oxfordshire; *Alien Gene Transfer in Crop Plants: Innovations, Methods and Risk Assessment* and *Alien Gene Transfer in Crop Plants: Achievements and Impacts*, both published by Springer, New York. He is also a recipient of the prestigious Norman E. Borlaug International Agricultural Science and Technology Fellowship. He is an acknowledged speaker and has several awards to his credit.

Dr. Shiv Kumar is Food Legumes Coordinator and works as Lentil and Grasspea Breeder at the International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat platform, Morocco. Before joining the present position, he served the Indian Council of Agricultural Research as a Plant Breeder for 18 years. He also served the International Crops Research Institute for Semi-Arid Tropics as Post Doctoral Fellow and worked on basic studies in chickpea breeding and genetics between 1991 and 1993. His post-doctoral work has led to identification of extra early photo-thermo insensitive genotypes in chickpea which have been used as donors across the globe. Dr. Kumar has over 25 years of research experience on basic and applied aspects of breeding rice and pulses including chickpea, grasspea, *Vigna* crops and lentil. He has been associated in the development of 28 varieties of pulse crops and one variety of rice. He also identified useful new germplasm for use in breeding program of rice, lentil, chickpea, grasspea, mungbean and urdbean. He has to his credit more than 300 articles including 110 research papers in refereed journals, 52 book chapters, 6 books, 7 technical bulletins and 2 training manuals. He also received a number of academic distinctions and awards including Rockefeller Fellowship, Best Scientist Award from IIPR for the years 2005 and 2008, and Best Research Team Award from MULLaRP of ICAR in 2008. His research interests include pre-breeding activities, genetic enhancement through conventional and marker-assisted breeding and biometrical genetics.

Plant Phenomics: An Overview

1

Jitendra Kumar, Aditya Pratap, and Shiv Kumar

Abstract

Precise and accurate measurement of traits plays an important role in the genetic improvement of crop plants. Therefore, a lot of development has taken place in the area of phenomics in the recent past. Both forward and reverse phenomics have been evolved, which can help in identification of either the best genotype having the desirable traits or mechanism and genes that make a genotype the best. This includes development of high throughput non-invasive imaging technologies including colour imaging for biomass, plant structure, phenology and leaf health (chlorosis, necrosis); near infrared imaging for measuring tissue and soil water contents; far infrared imaging for canopy/leaf temperature; fluorescence imaging for physiological state of photosynthetic machinery; and automated weighing and watering for water usage imposing drought/salinity. These phenomics tools and techniques are paving the way in harnessing the potentiality of genomic resources in genetic improvement of crop plants. These techniques have become much more advanced and have now entered the era of high throughput integrated phenotyping platforms to provide a solution to genomics-enabled improvement and address our need of precise and efficient phenotyping of crop plants.

Keywords

Phenomics • Forward • Reverse • Phenome • Phenes • Genomics • Genes

1.1 Introduction

Worldwide demand for crops is increasing rapidly due to rising global population, rising demand for biofuel and feed stocks and changing food preferences. Meeting future demand of agricultural production poses the greatest challenge to agricultural scientists and policy makers

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(Bruinsma 2003) because demand for cereals, biofuels and feed stocks has already surpassed the current supply and is expected to rise further in the near future (Furbank et al. 2009; Sticklen 2007). Therefore, there is a competition among crops for arable land in order to increase their production. Rising global mean temperature by 0.8 °C since the 1850s, which is expected to increase further by 1.8–4.0 °C by the end of this century, will have further impact on agricultural production due to changing climate (Solomon et al. 2007) and prevalence of abiotic stresses with more intensity and frequencies (Tester and Langridge 2010). It has been estimated that in future average crop yields may decline across Africa and South Asia by 8 % by the 2050s (Knox et al. 2012). These declines in yields have been predicted about 17 % in wheat, 5–16 % in maize, 11–15 % in sorghum, and 10 % in millet across above regions under regimes of climate change (Wheeler and von Braun 2013). Therefore, development of 'climate-smart' germplasm would be a priority to tackle these future challenges of climate change (Ziska and Bunce 2007; Leakey et al. 2009).

The use of conventional plant breeding methods has made substantial gain in crop yield worldwide. However, researchers are now observing that current breeding methods will not be sufficient to meet the projected future demand of foods (Furbank et al. 2009; Tester and Langridge 2010; Sticklen 2007). Therefore, this has shifted our focus towards the use of genomics and gene technology advances for assisting the current breeding programs in order to increase grain yields. These developments are being utilized in trait discovery, genetic dissection of complex traits and discovery of associated genes and their deployment in varieties. This has resulted so far in more than 5,000 publications on mapping of quantitative trait loci (QTL) and their isolation during the past years (Zamir 2013). In spite of these efforts, the identified QTLs/genes could not be deployed in mainstream breeding programs because identification of most of these QTLs/genes was not based on the precise and accurate phenotyping data of targeted traits. Hence, association of these QTL/genes with the phenotype in a 'real world' environment remains elusive as many false positive QTL have been reported earlier.

Although a large collection of germplasm of different crop species are available worldwide, phenotypic descriptions of these genome wide knockout collections are still limited. As a result, it restricted the use of genomic resources for identifying the allelic variation for a promising candidate gene in natural germplasm collection (see Miyao et al. 2007). The poor utilization of genomic resources could also be due to the lack of analysis of invisible traits and sometimes complex phenotypic effects of genetic modification. Therefore, identification of a candidate germplasm that carries genes for targeted traits is only possible when we will have the precise and accurate phenotyping profile of the germplasm. Phenotyping of valuable agricultural traits such as grain yield, abiotic stress tolerance, and nutritional quality is widely recognized as the most laborious and technically challenging because replicated trials are necessary across multiple environments over a number of seasons. Some of the current phenotyping tools also require destructive harvesting at fixed time intervals or at a particular phenological stage and are slow and costly. These bottlenecks in field phenotyping have driven intense interest over the past decade and hence efforts have been made on development of new high throughput phenotyping tools and techniques such noninvasive imaging, spectroscopy, image analysis, robotics and high-performance computing for phenotyping. These tools can not only be used in laboratories but also in field leading to high-throughput analysis of phenotypes in natural conditions as well as under controlled-environment conditions. Now, field evaluation of plant performance is much faster, and facilitates a more dynamic, whole-of-lifecycle measurement less dependent on periodic destructive assays. The dedicated high throughput controlled-environment facilities have also improved the precision in recording the data and reduce the need for replication in the field. Thus these advances have revolutionized the field of the accurate and precise phenotyping for

important traits and bring us to the age of 'phenomics' and overview of these developments have been presented in this chapter.

1.2 Origin of Plant Phenotyping

Plant phenotyping has been a part of crop and variety selection since the time of human civilization when humans selected the best individuals of a crop species for domestication (Diamond 1997). Subsequently it has become common practice in plant breeding for selecting the best genotype after studying phenotypic expression in different environmental conditions and also using them in hybridization programs in order to develop new improved genotypes (Pearson et al. 2008; Fisher 1925; Annicchiarico 2002). Ecologists used phenotyping to study phenotypic plasticity of genotypes during the middle of the twentieth century and suggested the role of the genotype and environmental conditions in the expression of plant phenotypes under which it develops (Suzuki et al. 1981). Subsequently, developments in ecology in relation to phenotyping are the trait-based approaches, in which phenotypic characteristics of a wider range of different species are evaluated either in the field (Reich et al. 1992) or under laboratory conditions (Grime and Hunt 1975; Poorter et al. 1990). They were used to derive different strategies by which the ecological niche of species could be described (Grime 1979) and to analyze the interdependence of various traits (Wright et al. 2004).

1.3 Phenomics

The word 'phenome' refers to the phenotype as a whole (Soul 1967) i.e., expression of genome for a trait in a given environment while in phenomics we get high-dimensional phenotypic data on an organism at large scale. Actually phenomics is used as analogy to genomics. However it differs from genomics. In genomics, complete characterization of a genome is possible while in phenomics, complete characterization of phenome is difficult due to the change in the phenotypic expression of traits over the environmental conditions (Houle et al. 2010).

1.4 Phenotype vs Phenomics

Phenotype of a plant can be described on the basis of morphological, biochemical, physiological and molecular characteristics. Different parameters are measured to describe these characteristics. Johannsen (1911) has coined the terms 'genotype' and 'phenotype'. He demonstrated substantial variation in quantitative traits to which he called 'phenotypical' in genetically-identical material and thus proved that variation in a given observed traits is not controlled entirely by genetics. Therefore, use of statistical analysis has been suggested for identifying the differences among genotypes because phenotypic variation within a genotype can obscure phenotypic differences among genotypes. This leads to origin of pheno- word. After 1950, 'phenotyping' as a noun, 'to phenotype' as a verb and 'phenome' as the collective noun were introduced, which have been accepted scientifically and are being utilized commonly in literature.

1.5 Forward and Reverse Plant Phenomics

Plant phenomics is the study of plant growth, performance and composition. Figure 1.1 showed the use of forward and reverse phenomics in genetic improvement. Forward phenomics uses phenotyping tools to discriminate the useful germplasm having desirable traits among a collection of germplasm. This leads to identification of the 'best of the best' germplasm line or plant variety. Use of high-throughput, fully automated and low resolution followed by higher-resolution screening methods have accelerated plant breeding cycle by screening a large number of plants at seedling stage. Thus interesting traits can be identified rapidly at early stage and there is no need to grow plants up to the

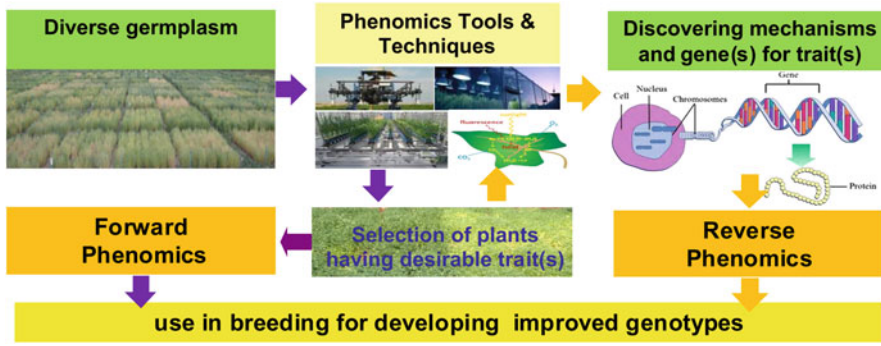

Fig. 1.1 Forward and reverse phenomics for genetic improvement in crop plants

maturity stage in field. Now it is possible in forward phenomics to screen thousands of plants in pots running along a conveyor belt, and travelling through a room containing automated imaging systems such as infra-red or 3D cameras. The pots are labelled with barcodes or radio tags, so that the system can identify which pots contain plants with interesting traits. The selected plants can then be grown up to produce seed for further analysis and breeding.

The reverse phenomics is used where the best of the best genotypes having desirable trait(s) is already known. Now through reverse phenomics, traits shown to be of value to reveal mechanistic understanding are dissected in details and subsequently the identified mechanisms are exploited in new approaches. Thus in reverse phenomics, we discover mechanisms which make 'best' varieties the best. This can involve reduction of a physiological trait to biochemical or biophysical processes and ultimately a gene or genes. For example, in case of drought tolerance, researchers try to work out the mechanisms underlying the drought tolerance and find out the gene or genes that are responsible for it. These genes are screened in germplasm or the gene can be bred into new varieties.

1.6 Genes and Phenes

To describe phenotype is more challenging than genotype because it changes over the environments. Therefore, the term 'phenotype' is not completely straight forward (Mahner and Kary 1997) and it varies among the various sub disciplines of biology. Ecologists traditionally define phenotype as trait when they refer to a phenotypic variable of a plant such as the specific leaf area (SLA). However, some ecologists also refer to traits in relation to characteristics of vegetation, such as the leaf area index (LAI). Like gene, 'trait' has been designated as 'phene'. However it can be over simplification for a one-to-one relationship between gene and phene because one gene can have a range of pleiotropic effects and many genes can control a trait. The term 'phenome' is being utilized as a counterpart to 'genome'. Thus as total constellation of all genes (alleles) present in an individual is known as genome. Therefore, similarly the phenome would be the aggregate of all the expressed traits of an individual. Actually, use of various terminology may overlap as they fulfill various and different needs for different niches of the scientific community. A clear and singular definition throughout the full domain of biology is desirable but probably unreachable (Mahner and Kary 1997).

1.7 Advances in Phenomics

Morphological, physiological and biochemical traits are important to breeders for making genetic improvement for yield, quality and tolerance to biotic and abiotic stresses. These traits have been discussed in details in Chap. 2.

Conventionally, phenotyping data on these traits are recorded either visually or manually, which is time-consuming and required a lot of efforts. This also increases chance of errors in measurement of traits. As a result, it increases chance to identify the false positive alleles, which leads to slow gain in genetic improvement. Therefore during the past few years, focus has been shifted on precise, accurate and rapid phenotyping of traits on a large scale. High-throughput phenotyping using non-invasive imaging technologies is a rapidly advancing field (www.plantphenomics.org.au; Furbank et al. 2009; Finkel 2009; Jansen et al. 2009; Berger et al. 2010). These techniques are based on colour imaging for biomass, plant structure, phenology and leaf health (chlorosis, necrosis), near infrared imaging for measuring tissue water content and soil water content, far infrared imaging for canopy/leaf temperature, fluorescence imaging for physiological state of photosynthetic machinery and automated weighing and watering for water usage imposing drought/salinity conditions. These advanced phenotyping techniques have been discussed in details earlier in a number of reviews (see Furbank and Teste 2011; Walter et al. 2012). The genotypes capable of maintaining stomatal conductance under salt induced osmotic stress have been selected successfully at the young seedling stage in wheat and barley using infrared thermography (Sirault et al. 2009). This technique has also been suggested to use for high-throughput seedling screening for drought tolerance in the vegetative stages of crop development and has great potential for low-cost, high-throughput field phenotyping. The genotypes having better photosynthetic capability and higher water use efficiency in field can be screened by measuring the canopy temperature using handheld hermopile based infrared thermometers (i.e. canopy temperature 'guns'). Chlorophyll fluorescence analysis has been used to test the maintenance of photosynthetic function under biotic and abiotic stresses leading to identification of resistance and susceptible genotypes. For this purpose, a commercial instrument namely pulse amplitude-modulated (PAM) or fluorometry has been developed which is based on fluorescence parameter measured in stress (Baker 2008). It can be used on whole leaves or small plants. It used successfully for abiotic stresses screening in Arabidopsis and tobacco (*Nicotiana tabacum*) or seedlings of dicots such as canola (*Brassica napus*) or cotton (*Gossypium* ssp.) (Baker 2008; Woo et al. 2008). It can also be used to determine projected leaf area and hence the growth rate if measurements are taken regularly over time (Barbagallo et al. 2003). The chlorophyll fluorescence images of the affected area of the leaf allow the early detection of disease symptoms caused by the pathogens. These infected areas can be quantified leading to identification of the susceptible and resistant response to pathogen attack, at least in the case of mildew on barley leaves (Swarbrick et al. 2006; Chaerle et al. 2009). Leaf spectroscopy or hyperspectral reflectance spectroscopy using radiometric or, more recently, imaging sensors are another established optical techniques related to chlorophyll fluorescence, which have been developed to study the stress related phenomics (Jones and Vaughan 2010). However, its use in plant breeding is limited due to difficulties in interpreting canopy temperature data.

Digital imaging is one of the least complicated but useful methods for quantitatively determining the stress tolerance. It is popular approach for in situ crop phenotyping in controlled environment facilities. It uses to take the digital images of growth over a period of plant development and measures quantitative changes in images caused by the sum of stress response mechanisms. In addition to this, taking digital images in visible wavelength regions also give opportunity to identify color of the plants. As a result, it enables to quantify senescence arising due to nutrient deficiencies or toxicities, or pathogen infections. It has been used successfully to quantify toxicity of germanium (as a toxic analogue of boron) in a mapping population of barley (Schnurbusch et al. 2010) and identified a QTL at the same locus as previously identified for boron tolerance using a visual score of symptoms (Jefferies et al. 1999). The attempt was also made to measure the water use

efficiency in plants (Harris et al. 2010). Use of non-destructive imaging using fluorescence and hyperspectral reflectance offers great promise in quantitative scoring of such adult plant resistance phenotypes. However use these techniques for screening biotic stresses is still limited.

1.7.1 Development Towards the Phenotyping Machines

ring the past one decade, vast amount of genomic resources have been developed and rapid development in genome sequencing has increased the genomic data bases such as, e.g. GABI DB or TAIR DB in model plant species and crop plants (Meinke et al. 1998; Riano-Pachon et al. 2009; Huala et al. 2001). High throughput genotyping platforms have increased the speed of genotype selection in breeding programs (Langridge and Fleury 2011). However phenotyping for complex traits related to anatomy, morphology, physiology and development is still less advanced, although high-throughput phenotyping techniques have increased our detection ability substantially at subcellular level for protein interactions or metabolism (Houle 2010; Kolukisaoglu and Thurow 2010). For plant breeders, screening component traits contributing to yield under field conditions at large scale is more important for making genetic improvement, but it is still lacking (Furbank and Tester 2011). However significant efforts have been made towards the development of automated phenotyping platforms during the past years (Granier et al. 2006; Jansen et al. 2009; Furbank and Tester 2011; Delseny et al. 2010; see Chap. 18) by taking advantages of throughput phenotyping facilities developed in the field of drug discovery, development, and animal behavior (Mayr and Bojanic 2009; Noldus et al. 2001). In brief, these platforms are equipped with sensor or image based systems under the controlled growth leading to establishment and implementation of the non-destructive imaging approaches for phenotyping (Furbank and Tester 2011; Fiorani et al. 2012). In these platforms, we can measure the plant size and leaf area of large germplasm collections using 2D color images and dense canopy by using 3D image technology and magnetic resonance imaging (MRI) (Poorter et al. 1988; Dornbusch et al. 2012). The fluorescence and hyperspectral analysis allow evaluation of various plant traits in a fast and non-destructive manner to characterize the leaves and roots at physiological or biochemical level. However, only specific aspects of plant functioning can be evaluated in this way. An exciting new development is the robotised sensor-actor for destructive sampling of relevant plant parts has widen the phenotyping capabilities by automated measurement of cellular processes and/or gene expression at specific time points (Alenyà et al. 2012). Relevance of a laboratory and greenhouse phenotyping technique is actually tested in field because traits considered critical in the greenhouse may be less important in the field. For example, the canopy of a stand is more relevant than of a single plant under field conditions. Therefore, mobile platforms such as a tractor equipped with specific sensors enabled larger spatial flexibility have been developed for the mechanistic field phenotyping measurements with high accuracy and repeatability in given plots, while drones or airborne platforms can cover vast agricultural areas. Though multi- and hyperspectral technologies (Rascher and Pieruschka 2008; Comar et al. 2012) can be used to analyze physiological process, only few robust techniques such as the laser-induced fluorescence transient (LIFT) approach are available to estimate photosynthetic efficiency in the field (Pieruschka et al. 2010). Dedicated field sensors are already applied in precision agriculture for nutrient management (Scotford and Miller 2005) and may become important tools for sensing of plant disease in the near future (Mahlein et al. 2012). Establishment of wireless sensor networks enables continuous monitoring of the environment and crop properties and will provide valuable information for agricultural management (Ruiz-Garcia et al. 2009).

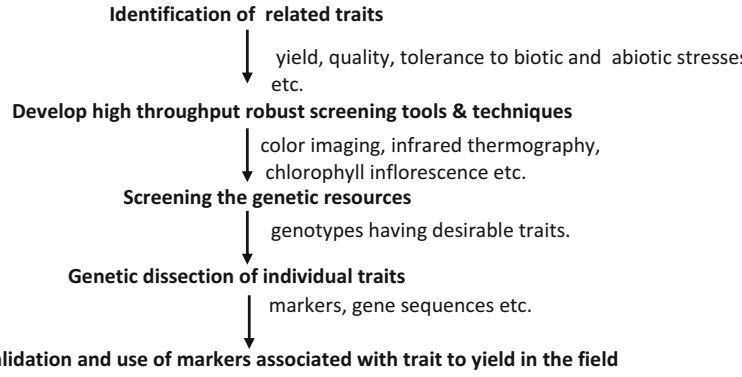

Fig. 1.2 Flow chart of application of non-destructive phenotyping in genetic dissection of trait

1.8 Harnessing the Potentiality of Genomics Through Phenomics

Vast amount of genomic resources are available in public domain but these could not be utilized with their potentially due to the lack of precise, accurate and high throughput phenotyping tools and techniques. Therefore, efforts have been made for the development of high throughput phenotyping tools and techniques for screening of morpho-physiological traits related to biotic and abiotic stresses. The genomic resources developed in a plant species can be linked with physiological and morphological data collected using current phenotyping approaches available at automated phenotyping platforms worldwide. These high throughput phenotyping tools collect the precise and accurate observations and allow analysis of data for understanding the whole phenome of the plant under a wide range of environmental conditions. Thus like genomic platforms, phenotyping platforms develop databases such as the plant meta-phenomics database (Poorter et al. 2010) or the Plant Trait database TRY (http://www.try-db.org, accessed September 2012) which bring together phenotypic responses to the environment for a wide range of plant traits and parameters. These phenotyping database along with available international genomic databases (TAIR, TIGR and NCBI, and with other 'omics' information such as metabolomic, proteomic and transcriptomic data) have now become important to understand the genetic architecture of complex traits. Phenomics has not only allowed to dissect the complex traits through genomics but also helped to use genomic resources in discovering new genes/QTL, identification of function of a gene sequence and helped to increase the genetic gain for traits having low heritability (see chap. 17 for details). This understanding will allow us to simulate and predict plant properties in particular of complex traits such as yield or biomass, the most important challenge to address future needs of a growing human population. Both forward and reverse phenomics approaches can be used to harness the potentiality of genomic resources. The accurate, cost-effective, high-throughput phenotyping is pivotal to fine mapping of traits, regardless of the genetic approach for producing allelic recombination or assessing variation by re-sequencing technologies. Phenomics can be used in reverse genetic studies and can help to identify the function of a particular gene(s) in growth and development of crop plants and can be used to identify the allelic variation to target the associated genes (Fig. 1.2).

1.9 Conclusion

For making successful genetic improvement in crop plants, plant breeders first identify the desirable genotypes having target traits by screening a collection of germplasm accessions. These target traits then are combined together through hybridization. This cycle of selection-hybridization-selection has been implementedon the basis of visual observation since

domestication of crop plants. Though visual screening is easy and precise for qualitative and highly heritable traits, its use is less precise for quantitative traits and those traits, which are difficult to observe visually (physiological and biochemical traits). Moreover, vast amount of genomic resources have been developed in a number of crop species in the past. The available gene sequences and molecular markers could still not be associated with any traits due to the lack of phenotyping of germplasm collections. For utilizing these genomic resources and identification of desirable plants, the precise phenotyping of germplasm accessions for challenging traits is required in various crop species.

In the recent past, various techniques and methodologies have been developed for screening biotic, abiotic, physiological and biochemical traits in crop plants. These technologies have become very advanced in the era of digital science. These plant phenomics developments are actually helping to make simply plant physiology in 'new clothes'. Thus this trans-disciplinary approach promises significant new breakthroughs in plant science. Phenomics provides the opportunity to study previously unexplored areas of plant science, and it provides the opportunity to bring together genetics and physiology to reveal the molecular genetic basis of a wide range of previously intractable plant processes. The challenges ahead in plant-based agriculture will require the scale of quantum advances we have seen in information technology in the past 20 years and we need to build on these advances for security of global food, fiber and fuel.

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Traits for Phenotyping

2

Engin Yol, Cengiz Toker, and Bulent Uzun

Abstract

The term plant phenotyping has been regenerated with the contribution of sensors, system technologies, and algorithms. This new plant describing concept allows multi-trait assessment with automatic measurements. Uniform structure, nondestructive measurements, precise results, and direct storage are the advantages of digital phenotyping. The hyperspectral spectroradiometers and imaging technologies lead the way of new plant phenotyping applications. This high-throughput technique therefore requires lots of traditional and novel traits to present new characterization. Digital-based phenotyping in plants is new and still a developing area of research. The most often used traits of digital phenotyping are canopy temperature, chlorophyll fluorescence, stomatal conductance, chlorophyll content, leaf water potential, leaf area, fruit color, carbon isotope discrimination, light interception, senescence, and root traits which have been discussed in this chapter together with their advantages, limitations, and plant breeding potentials.

Keywords

Trait • Characterization • Phenomics • Phenotyping • Imaging • Plant breeding

2.1 Introduction

Plants are fundamental to life, providing the basic and immediate needs of humans for food and shelter. Domestication of plant species is an important step in the human history for food uses and diversification. Three steps were proposed for plant domestication: (i) collecting seeds from their native habitat and planting them in areas where they were perhaps not adapted as well, (ii) inhibiting certain natural selection pressures by growing the plants in a field under cultivation, and (iii) applying artificial selection pressures by choosing characteristics that would not have necessarily been beneficial for the plant survival (Xu 2010). Selection pressure includes changes in allele frequency, gradations within and between

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species, fixation of major genes, improvement of quantitative traits (Tanksley and McCouch 1997; Xu et al. 2010), and resulting in selection of desirable traits (Harlan 1975).

Identification of plant characteristics contributes to germplasm utilization and thereby characterization is an important step in genetic resource management (Bioversity 2009). Traits are the most important tool for describing accessions and these plant characteristics are used for diagnostic objective to depict the crops as accession and discriminate them from one another (Bioversity 2009). Traditionally, crops are characterized by measuring phenotypic traits such as color of plant parts and quantitative traits like seed yield, drought tolerance, etc. (Rao 2004). Crop phenotypic traits can be quickly, easily, cheaply, and nondestructively observed or measured in field (Torres and Pietragalla 2012).

Although agronomic traits have been used extensively in agriculture and biochemical, molecular, and also high-throughput phenotyping, regardless of background of traits, all must have critical features such as simple to score, specific to species, precise and uniform, universal, easy, and quick discriminative.

2.2 Traditional Phenotyping

Till the last decade, plant phenotyping was done with traditional methods using agro-morphological traits which have been used in characterization and interpreted to two major data as qualitative and quantitative. Qualitative data is used for diagnosis of highly heritable traits that is not influenced by environmental fluctuations. The traits are scored easily, providing fast discrimination between germplasm, and are generally regulated by major genes (Bioversity 2007). The other data is quantitative, used for traits, which are expressed by the gene interactions and also highly affected by genotype and environment interactions (G × E). Both qualitative and quantitative data are scaled to degree of traits expression. These scales should be nominal, ordinal, continuous, and binary. In qualitative traits, nominal scales are numbered to define the traits by names or labels (color, shape). Ordinal scales are similar to nominal type but have order. Binary scales have only two types of diagnosis as yes/no or absent/present. On the other hand, quantitative traits are recorded by measuring, counting or weighing, and using continuous scales (countable data, such as "1,000-seed weight") (Bioversity 2007). Some traits can be expressed in degrees, which are then recorded on a scale (from 1 to 9). This is valid especially for resistance or susceptibility to different types of biotic and abiotic stresses (Jaramillo and Baena 2002).

Although many of qualitative and quantitative traits have been identified for different crops, general concept of plant breeding has focused some major traits. Improvement of agronomic performance, tolerances or resistances to biotic and abiotic stresses and quality traits are main instruments in plant breeding (Riley et al. 1996). The traits related to agronomic performance contain yield and yield components. Yield concept may change according to crops and aim of utilization. Seed quantity in cereals and legumes is assessed as a yield trait. Fiber is evaluated as a yield trait in fiber crops, although it requires manipulation for industrial uses. Vegetable oil quantity is a yield criterion in oilseed crops. Vegetative parts are evaluated as yield trait in forage crops. Yield is therefore not only seed yield but also different part of plants and processes of seed in certain crops. Quality parameters may contain nutritional and flavor traits such as high oil content in oilseed crops, high oleic acid level in vegetable oils, high protein content in legume crops, and smell, color, and flavor in fruits and vegetables. Tolerance or resistance to biotic stresses includes traits imparting disease and insect resistance. Disease resistance occurs against fungi, viruses, bacteria, and phytoplasmas. Tolerance or resistance to abiotic stresses is generally related to drought, heat, cold, salt, soil conditions, and light. Resistance and tolerance to biotic and abiotic stresses are explained with scales. However, identification of tolerance level is highly variable and requires experience.

Agro-morphological traits should be universal. If not, different and inconsistent characterization might lead to misunderstanding for breeding studies. One type of characterization in a population is therefore better than the individual plant assessment. Plant traits have been catalogued by International Plant Genetic Resources Institute (IPGRI) and different international plant research organizations for successful characterization of plant genetic resources. This definitive scheme was named as "descriptor" to provide "one language" understanding about plant characteristics. It contains passport data and descriptive features such as genus, species names, sowing date, leaf and flower color, and intensity of coloration and pre and postharvest traits. Four categories viz., passport descriptors, management descriptors, characterization descriptors, and evaluation descriptors have been generated to recognize plant genetic resources. Passport descriptors provide information on systematic and provenance, whereas management descriptors include data on where the material is stored, quantity of a material stored, and its condition. Characterization descriptors are related to heritable traits which are independent of the environment (Summerfield et al. 1996). Hundreds of crop plants have been catalogued by the group of Bioversity International. Research centers of FAO such as ICRISAT in India and ICARDA in Syria and national institutes such as NIAS in Japan, GENBANK.AT in Austria, NCGR in Indonesia, and CIRAD in France have also published descriptors for different plants.

2.3 Modern Phenotyping

Genotype in plants includes all of genes. Phenotype is a combination of genotype and environment. Phenome is gene and environment interaction or the expression of the genes thanks to characteristics in existing conditions (Furbank and Tester 2011). Phenomics is a biological term that is related with the measurement of phenomes (Bilder et al. 2009). Plant phenotyping is a rapidly evolving concept that the phenotype develops while the plant growing from the association between the plant genetic background and the environment in which the plant develops (www.fz-juelich.de).

The scientific accuracy of molecular breeding is strongly related to phenomics and therefore phenotyping (Xu and Crouch 2008). The main aim of phenomics is to connect between genetic, plant function, and agricultural characteristics (Furbank and Tester 2011). Phenomics is an important concept because only agro-morphological or molecular data are inadequate for analysis of plant natural structure due to the fact that the connection between genes and phenotypes is particularly weak in studies of crop × environment. Phenotyping provides association among genotype-phenotype-environment to enable sustainable and efficient crop production by taking into consideration the climate change and changing agricultural production conditions (Tardieu and Schurr 2009). Plant phenotyping is therefore necessary to elucidate the functional role of gene networks under real (natural) conditions and understand the impact of biological diversity in adaptation of plants to heterogeneous and fluctuating environments and devise strategies to anticipate global climate changes (www.fz-juelich.de).

Phenotyping offers opportunity for functional studies on special genes, forward and reverse genetic analyses, and development of new crops with beneficial characteristics (Berger and Tester 2009). In the last decade, high-throughput phenotyping platforms have gained popularity, which are capable of handling several thousand of plants in a single study under controlled environmental conditions and allowing high-accuracy phenotyping (Tardieu and Schurr 2009). Using advanced technology, many traits are assessed with high-throughput phenotyping. Large scale and large amounts of data can therefore be obtained in high-throughput analysis which allows phenotyping mutants, mapping and breeding populations, and germplasm collections under different growth conditions. Large numbers of proteins and metabolites could be analyzed with high-throughput phenotyping without any necessity of tissue

extraction. Physiological measurements such as photosynthesis, nutrient uptake, plant growth and development process, and measurements in fields can be made possible via high-throughput phenotyping (NIFA-NSF Phenomics Report 2011). Recently, field phenotyping platforms have been developed and they can control growing period, gas transmission, and status of canopies with large number of plants and genotypes, by using imaging techniques with sensors placed on field vehicles or flying platforms (Tardieu and Schurr 2009).

2.4 Techniques for Phenotyping: Trends

Plant phenotyping has been carried out by farmers and breeders for ages. In the traditional phenotyping, morphological traits are assessed with statistical analysis which has to be done manually. It needs human efforts, time, and resources to measure plant characteristics. Performing of sensor technologies and algorithmic applications for automatic phenotyping are being handled to overcome the defect of the manual techniques (Klose et al. 2009). These digital techniques provide multi-trait assessment with automatic measurements and saving time. Uniform structure, nondestructive measurements, precise results, and direct storage are also the advantages of digital phenotyping.

The hyper-spectral spectroradiometers is one of the digital techniques which allows to formulate different indices and then to infer a wide array of morphological and physiological traits of plants (http://maizephenotyping.cimmyt.org/index.php).

Spectral reflectance of plant architecture enables monitoring of several dynamic complex traits in phenotyping. Field spectrometers (and spectroradiometers) are used to measure spectral reflectance in ranges of 350–2,500 nm (Nasarudin and Shafri 2011). The physiological changes of a crop canopy including chlorophyll content, photosynthetic capacity, nitrogen and plant water status, and carotenoid content are measured with spectral reflectance. These values obtained by spectral reflectance allow estimation of the green biomass, photosynthetic area of the canopy, measurement of photosynthetic radiation absorbed by the canopy, and canopy architecture. Grain yield has also been estimated using spectral reflectance indices during different developmental stages of crops (Fender et al. 2006; Yazdanbakhsh and Fisahn 2012; O'Shaughnessy et al. 2011; Mullan and Mullan 2012).

Digital imaging analysis provides a rapid way of precisely measuring plant features (Tuberosa 2011). This technique is the most significant technology of plant phenotyping. Different imaging systems have been developed up to date.

Spectral imaging is one of the important imaging systems which provide reliable spectral information under chaotic outdoor conditions. A video camera is used for plant detection (http://maizephenotyping.cimmyt.org/index.php). An on-the-screen-display generates a video sequence and includes the measured data. As a consequence, the data can be interpreted together with an image and a clear correlation (http://maizephenotyping.cimmyt.org/index.php). Plant height is an important trait for imaging technology as plant shape is well characterized with this method (Fender et al. 2006).

In three-dimensional (3D) imaging, digital photos of the top and sides of plants are combined into a 3D image. Technically, pots of plants move on a conveyor belt through an imaging chamber and 3D models are automatically generated by a computer program (http://www.plantphenomics.org). Obtained images are transferred to the software and required editions as color improving and optimization are made. Digital images have advantages such as simple recording, transmitting, and storing in a database. However, algorithms are necessary to gather and analyze the huge amount of data (Tsaftaris and Noutsos 2009). *Arabidopsis thaliana* has been used as a model plant for development of imaging systems.

Thermal imaging is a practical alternative to specific measurements, because temperature of canopy of the field can be analyzed in a short time and outlines can be produced for the traits in the field (Cohen et al. 2005). Thanks to thermal imaging techniques, the water status at variety

level can be defined under different environmental and greenhouse conditions (Grant et al. 2006). The thermal measurements mostly rely on evaporation, with high and low level of temperature reflecting stomatal structure as closing and opening, respectively (Chaerle et al. 2007). In thermal imaging, closing stomata is an early and sensitive reaction of plants facing drought stress which is a cause of reduced transpiration. Leaf temperature locally rises and produces a spatial temperature pattern that can be visualized by thermography (http://maizephenotyping.cimmyt.org/index.php). Thermal imaging systems enable fast and rapid collection of data on a single leaf or a canopy area (Grant et al. 2007). This system also provides great number of crop measurement with low cost.

In fluorescence imaging, plant health and photosynthetic activity can be characterized. Fluorescence occurs when an object absorbs light of one wavelength and gives off light of a different wavelength, which a computer program converts the resulting fluorescence into false-color signals to allow instant analysis of plant health (http://www.plantphenomics.org). Magnetic resonance imaging (MRI) allows studying plant roots. It enables the 3D geometry of roots to be viewed just as if the plant was growing in the soil (http://www.plantphenomics.org). Near-infrared (NIR) imaging provides detailed data on the watering status of leaves (Eberius 2008). Phenonet sensor network, phenomobile, phenotower, and blimp are important tools which allow plant phenotyping in the field to study lots of plants, simultaneously (http://www.plantphenomics.org).

2.5 Traits for Phenotyping: Trends and Options

In current plant phenotyping, digital-based systems and sensor technologies (highly sensitive imaging, spectral imaging, robotics, and high-algorithmic calculation) allow a wide range of evaluation of complex traits such as yield, growing period, tolerance/resistance to diseases, architecture, and the fundamental quantitative parameters (http://www.lemnatec.com/plant-phenotyping.php). It is known that adaptive traits are complicate and multigenic, and understanding of their genetic structure is not completely known (Salekdeh et al. 2009). However, this complexity can be understood with new traits by evaluating their responses to environment. As plant phenomics is still a developing study area, new and most often used traits in phenomics are evaluated in this section, while other traits are described in Table 2.1.

2.5.1 Canopy Temperature

It is an important parameter detected by thermal imaging. In photosynthesis, stomata open to admit carbon dioxide and simultaneously vapor moves away from leaves, which cool the leaf surface (Roth and Goyne 2004). Under water-stress conditions, crop temperatures rise due to insufficient transpiration. Canopy temperature therefore affects water-use efficiency, stomatal conductance, photosynthesis activity, transpiration rate, leaf area index, sink strength, vascular capacity, and crop yield (Fischer et al. 1998; Pietragalla 2012). The measurement of canopy temperature can change according to canopy traits and stress tolerance. Thermal infrared thermometers are noninvasive and reliable for crop temperature measurements. Canopy temperature has been identified by canopy temperature devices and infrared thermometers, easily and at no extra cost. They may make non-homogenous measurements as dimensional between leaves, but they would be improved with replications, without damaging natural structure of the leaves (Grant et al. 2007). Other system is remote thermal imaging which supplies information as spatial of surface temperature and hence makes it possible to recognize of canopy temperature variation on wider areas (Alchanatis et al. 2010). Wireless infrared thermometer system is useful method for identification of canopy temperature. This measurement type was developed by O'Shaughnessy et al. (2011) who designed a model for field observation with wireless thermographic camera. Satellite and airborne imaging are also used for field trials.

Table 2.1 Other traits used in plant phenotyping

Trait	Reference
Biomass accumulation	Montes et al. (2007)
Internode length	http://www.lemnatec.com/plant-phenotyping.php
Osmotic adjustment	Pierre and Arce (2012)
Plant width	Phenofab (2012) (http://www.phenofab.com/)
Plant roundness	Phenofab (2012) (http://www.phenofab.com/)
Plant height	Fender et al. (2006)
Plant compactness	Phenofab (2012) (http://www.phenofab.com/)
Plant orientation	Phenofab (2012) (http://www.phenofab.com/)
Fruit shape	Scott (2010)
Fruit size	Phenofab (2012) (http://www.phenofab.com/)
Fruit netting	Phenofab (2012) (http://www.phenofab.com/)
Fruit cracking	Phenofab (2012) (http://www.phenofab.com/)
Leaf tracking	Phenofab (2012) (http://www.phenofab.com/)
Leaf relative water content	Mullan and Pietragalla (2012)
Leaf rolling	http://www.lemnatec.com/plant-phenotyping.php
Leaf angles	Phenofab (2012) (http://www.phenofab.com/)
Leaf length	Phenofab (2012) (http://www.phenofab.com/)
Leaf pigment content	Matsuda et al. (2012)
Leaf color	Phenofab (2012) (http://www.phenofab.com/)
Panicle color	Phenofab (2012) (http://www.phenofab.com/)
Panicle position	Phenofab (2012) (http://www.phenofab.com/)
Panicle area	Phenofab (2012) (http://www.phenofab.com/)
Germination rate	Phenofab (2012) (http://www.phenofab.com/)
Germination consistency	Phenofab (2012) (http://www.phenofab.com/)
Germination off-types	Phenofab (2012) (http://www.phenofab.com/)
Disease resistance	Phenofab (2012) (http://www.phenofab.com/)
Water-use efficiency	Pierre et al. 2012
Drought resistance	Phenofab (2012) (http://www.phenofab.com/)
Drought recovery	Phenofab (2012) (http://www.phenofab.com/)
Nitrogen status	Berger et al. (2013)
Nutrient-use efficiency	Phenofab (2012) (http://www.phenofab.com/)
Salinity tolerance	James and Sirault (2012)
Total mineral content	Benamar et al. (2013)

Airborne imagery has advantages compared to satellite system and it can screen the fields about 6,000–9,000 ft above horizontal area of field (Roth and Goyne 2004). Lastly, trees analyzed with frontal images by automatic system were able to detect canopy temperature, remotely (Jimenez-Bello et al. 2011). This technique was tested and canopy temperature indicated good correlation with plant water status. Also, the system is not required to control a reference image and cameras can take thermal and normal scenes, simultaneously. In different plants, canopy temperature imaging studies have conducted. Canopy and leaf imaging were tested for grapevine to test stress responses in various water regimes (Grant et al. 2007). Canopy water status and canopy temperature in different stress conditions were analyzed with high-throughput phenotyping in maize (Romano et al. 2011; Winterhalter et al. 2011; Zia et al. 2012). Infrared thermal imaging technology was used for identifying water-stress-tolerant maize genotypes of different phenology (Zia et al. 2012). As a result, selected spectral indices and IR-temperature indicated positive relationship with canopy water mass under different

drought stresses. Phenotyping supported by non-destructive high-throughput technology is therefore beneficial for identification of canopy water status. Canopy temperature has also been used for crop water status in cotton (Alchanatis et al. 2010), peach trees (Wang and Gartung 2010) and vineyard (Orbegozo 2012). There was a strong correlation between canopy temperatures and stomatal conductance and stem water potential. Other study about association of canopy temperature and stomata was conducted by Stoll et al. (2008) to image *Plasmopara viticola* effect in grapevines under various water availability regimes.

2.5.2 Stomatal Conductance

It is a trait related to leaf and canopy gas exchange. It responds rapidly to soil water status and controls photosynthetic activity and growth (Munns et al. 2010). Higher density, size, and degree of opening of the stomata allow greater conductance, and thus photosynthesis and transpiration rates are potentially higher (Pietragalla and Pask 2012). Stomatal conductance has high breeding value due to showing high heritability and providing high correlation with yield; greater leaf conductance under warmer temperatures has been associated with cooler canopy temperatures (Pietragalla and Pask 2012). The connection of stomatal conductance and yield in wheat was identified as positive in a 6-year study (Fischer et al. 1998). Stomatal conductance is also related to water deficits by the fact that as stomata close, leaf temperatures rise (Grant et al. 2007). The handheld porometer provides rapid measurement of leaf stomatal conductance in irrigated trials, though it is not a recommended measurement under water stress (unless very mild) as the stomata are generally close (Pietragalla and Pask 2012). Genetic diversity and stomatal changes to water deficit in field conditions can also be screened by infrared thermography (Munns et al. 2010). Stomatal conductance and canopy temperature were used to evaluate soil water potential in cotton (Padhi et al. 2012). The natural oxygen isotope composition (^{18}O) was used for observing stomatal conductance in leaves and seeds (Barbour et al. 2000). Measuring ^{18}O in plant material offers four advantages: (i) it provides an integrated measure of stomatal conductance and leaf temperature over the period that the analyzed tissue was formed; (ii) it avoids a number of experimental problems typical of measuring stomatal conductance; (iii) it allows for the collection of a large number of samples; and (iv) it requires very little labor in the field (Tuberosa 2011).

2.5.3 Chlorophyll Fluorescence

It is commonly used in phenomics to see the effect of different genes or environmental conditions on the efficiency of photosynthesis (http://www.plantphenomics.org). It is related to photosynthetic activity under stresses like salinity and drought. Imaging system is crucial because suitable fluorescence imaging provides information about the causes of the heterogeneity. The most common measurement was made with determining the photochemical activity of light harvesting in photosystem II. It was formulated as Fv/Fm. Fv is variable fluorescence from leaves adapted to darkness and indicates PSII ability to present photochemistry. Fm is maximal emission of electromagnetic radiation from dark- and light-adapted leaf and demonstrates level of fluorescence when primary quinone electron acceptor of PSII is maximally reduced (Baker 2008). Fv/Fm generally decreases when plants are exposed to stress in the light, and this event provides an easy and fast tool for observing stress (Baker 2008). This method is able to carry out whole-plant average measurements or to target leaves with high-throughput technology (Furbank and Tester 2011). Chlorophyll fluorescence can be measured by using hyper-spectral spectroradiometers like that radiometric and imaging sensors.

2.5.4 Chlorophyll Content

Leaf chlorophyll content has a positive relationship with dry root biomass (Songsri et al. 2009), seed yield (Puangbut et al. 2009), and tolerance to iron-deficiency chlorosis (Samdur et al. 2000). Nodulation and nitrogen fixation status could be identified by leaf chlorophyll measurements (Vollmann et al. 2011). It can be quickly measured using a portable optical meter (absorbance of red light at 650 nm and infrared light at 940 nm) (Mullan and Mullan 2012). A handheld portable SPAD chlorophyll meter has been used widely to estimate chlorophyll content (Dwyer et al. 1991). Using SPAD data, grain protein content; the alveogram parameters W, L, and P/L; and dry gluten content were predicted by Poblaciones et al. (2009). In St. Augustine grass, SPAD values had positive correlation with chlorophyll content, visual ratings, and total nitrogen (Rodriguez and Miller 2000). Image analysis should also be used for chlorophyll content determination after standardization in a field, which is important application area for high-throughput phenotyping (Vollmann et al. 2011). Leaf hue measurement was performed with image analysis by Majer et al. (2010) who found a significant correlation between leaf hue and chlorophyll content. In sugar beet, chlorophyll content was identified with "neural-network model" which is compact system based on RGB (red-green-blue) components of the color image taken by classic camera (Moghaddam et al. 2011). Chlorophyll measurement was supported by SPAD reading that was evident of neural-network model accuracy.

2.5.5 Leaf Water Potential

It is a trait related to plant water energy. Water in plants is transported within the xylem system under negative pressure which is positively related to the amount of water stress (Pierre and Gonzlez 2011). The water potential can be measured with chamber pressure method (Waring and Cleary 1967). With spatial thermal imaging, water potential can also be identified by canopy temperature. In cotton, Cohen et al. (2005) identified leaf water potential and crop water status by thermal images and spatial analysis, respectively. Spectral reflectance and canopy temperature were combined to evaluate leaf water potential in grapevine by Vila et al. (2011). The maps from the spectrometric techniques were similar to the maps of the observed leaf water potential in this study. The other study in grapevine was conducted by Lang et al. (2000) who used global positioning system (GPS) and digital remotely sensed images to map full spectrum leaf reflectance in vineyards. This work suggested that remote-image and leaf spectral reflectance analysis may be a strong tool for monitoring changes in metabolism associated with plant stress. Differently, near-infrared (NIR) spectroscopy technique is used as remote sensing study to predict plant water status in grapevine (De Bei et al. 2011). NIR spectroscopy was a new approach for fast and low cost of analysis of water potential. In Satsuma Mandarin, leaf water potential was exhibited with image analysis (Kriston-Vizi et al. 2003). Technically, an absolute reflection of mandarin leaf surface was calculated by comparing with known reference target reflectance and thus leaf water potential was estimated in this study. A study to measure leaf water potential of field-grown potato was conducted by Zakaluk and Ranjan (2008). Artificial neural-network (ANN) model was used to imaging with RGB digital camera. As a method, plants were selected randomly in plots and images from plots were classified following to calibration to isolate young green leaves from older ones. The obtained images and vegetation indices were converted with using PCA (principal components analysis). According to Zakaluk and Ranjan (2008), the ground-based digital camera is adequate to predict leaf water potential of potato plants in the frame of high-throughput plant phenotyping.

2.5.6 Leaf Area

All green surfaces, e.g., leaf lamina, sheath, stem, and spike of crops, relate to the light interception and photosynthetic potential, the surfaces for transpiration/water loss, and the above ground biomass (Pask and Pietragalla 2012). Leaf area measurements therefore have importance in studies of plant growth (Cristofori et al. 2007). Some instruments and prediction models such as drawing, blueprinting, photographing, conventional planimeter, and electronic leaf area meter can be used to measure leaf area (Gao et al. 2012). Image analysis with software should also be assessed as an alternative method (Cristofori et al. 2007). Digital camera imaging is fast and provides accurate analysis using appropriate software (Bignami and Rossini 1996). Leaf area index (LAI) is also a leaf area related trait indicating radiation intercepted by the canopy, and therefore it defines crop canopy photosynthetic activity (Xu et al. 2010). LAI is also a fine character enabling to determine plant transpiration and CO_2 exchange which are important to understand energy exchanges (Wu et al. 2008).

2.5.7 Fruit Color

The fruit color trait has commercial value in many crops. It should elucidate quality, texture, size, and flavor in many horticultural crops (Picha 2006). Many breeding programs have conducted to understand the genetic and physiological mechanisms of fruit color (Yoshioka and Fukino 2010). Traditionally, phenotyping of fruit color has been assessed visually; however, this type of evaluation might lead to variable results depending upon experience and training. Computer-based analysis of objects from digital images presents high-quality data and characterization (Darrigues et al. 2008). Different plant parts have been analyzed with digital phenotyping. The most popular color phenotyping studies have conducted in tomato and software (Tomato analyzer) was used by Darrigues et al. (2008) and Gonzalo et al. (2009). In this technique fruits are scanned and images are sent to software. In analyzing of color measurements, RGB colors are assessed as essential colors. Color test module is used to obtain RGB data and after then it is converted to the CIELAB color space which uses L, a, b codes to determine colors difference (Rodríguez et al. 2010). In fruit color phenotyping of melon, each fruit was scanned with full flatbed scanner with a black material cloth to hinder out-lighting and to enable homogeneous background (Yoshioka and Fukino 2010). Color signature method was used in this system and the obtained images were transformed to a statistical image data. The petal color of Begonia × tuberhybrida Voss was also evaluated by image analysis (Lootens et al. 2007). Petals were firstly photographed, and then according to flower color comparisons, varieties were divided into color groups as proposed by the manual of the RHS Colour Chart (Yoshioka et al. 2006).

2.5.8 Carbon Isotope Discrimination

Carbon isotope discrimination (Δ) is a trait implying the amount of ^{13}C used by photosynthetic activity (Khazaie et al. 2011). In photosynthesis, heavy isotope of carbon is discriminated which causes reduction of the dry matter in ^{13}C (Merah et al. 2001). It has positive correlation with the ratio of inner leaf CO_2 concentration to circumfused CO_2 concentration (Ci/Ca), therefore gives transpiration efficiency (TE) and long-range Ci/Ca ratio (Monneveux et al. 2005). In wheat (*Triticum aestivum* L.), high negative relation with TE and carbon isotope discrimination (Δ) proposed that low Δ increased TE and biomass under stress conditions (Rebetzke et al. 2002). In different crops, ^{13}C can be used for estimation of stomatal conductance and water-use efficiency under water-stress conditions (Tambussi et al. 2007). ^{13}C and seed yield had negative correlation in drought conditions, and therefore when biomass production is limited, low stomatal conductance

adds to drought tolerance (Turner 1997). Near-infrared reflectance spectroscopy (NIRS) was used to predict ^{13}C in wheat seeds by Ferrio et al. (2007). There were significant correlations between NIRS-predicted ^{13}C and measured ^{13}C. Δ has therefore been used as a monitoring machine for identifying variations in water-use efficiency (WUE) and the development of varieties with improved WUE and drought tolerance (Lopes and Mullan 2012).

2.5.9 Light Interception

It is an important function in photosynthesis processes (Sarlikioti et al. 2011) and has strong relationship with plant growth in agricultural crops and forest trees (McCrady and Jokela 1998). This trait is related to light-use efficiency model which includes two conceptual components: (i) photosynthetically active radiation (PAR) and (ii) light-use efficiency (LUE) (Tharakan et al. 2008). PAR interception is generally determined with a 0.8-m light rod and a reference sensor (Sarlikioti et al. 2011). Measurements are repeated for 5–7 weeks. Generally, light interception varies on crop development and dry matter accumulation and it generally decreases exponentially from top to bottom of canopy (Liu et al. 2012). It is usually Beer's law that has been generally used to determine the amount of light intercepted by a plant at canopy level and this method is on the basis of the use of leaf area index (Chenu et al. 2007). However, this method is inadequate because plant and canopy heterogeneity is ignored in light interception. In recent years, to analyze the genotype-environment interactions, several techniques have been developed for functional-structural plant models which combine physiological processes with three-dimensional structure of crop plants (Sarlikioti et al. 2011). This model was used by Chenu et al. (2007) that was expressed as a new procedure to enhance light interception which has a strong relation with biomass production and yield.

2.5.10 Senescence

It is an essential part of a plant's lifecycle related to physiological processes while the plants mature (Edwards et al. 2012). It can be observed in different parts of a plant: roots, germinating seeds, and reproductive organs. Leaves are used for senescence studies. Generally, leaf senescence should be evaluated at organ level; however, it is also associated with cellular or organismal death (Lim et al. 2007). Leaves loose chlorophyll during senescence as chloroplasts differentiate into pigmented plastids (Thomas 2012). For identification of crop senescence, regular assessment of the proportion of the canopy that is green and non-green is important and can be determined by visual assessment. Spectral reflectance, visual imaging (Howarth et al. 2011), and nuclear magnetic resonance (NMR) methods (Leport et al. 2011) have also been developed for phenotyping of leaf senescence.

2.5.11 Roots

Roots play a critical role in plant growth and development. Water and nutrients, which are indispensible for plant's life, are absorbed by the roots. They store carbohydrates and other substances and enable biosynthesis of hormones which are required for growth (Zhu et al. 2011). Root has a three-dimensional complex system which contains lots of standard traits (root length density, amount of root, root size, total root surface, root length, root growth response, average root radius and root length, number of lateral roots, etc.) and new traits (network perimeter, solidity, convex area) (Weitz 2009). This architectural structure shows differences according to species and environmental effects. Age is also important for root structure; mature crops have more complicated root system than the young crops. Therefore, changes in root structure might affect yield and stress-tolerant traits (Iyer-Pascuzzi et al. 2010). Excavation of roots or washed soil cores has been used for

measurement of root traits to understand the root architecture. However, these measurements show differences due to environmental conditions (Hund 2010). On the other hand, high-resolution phenotyping provides new techniques to analyze root structure in laboratory and greenhouse conditions (Topp and Benfey 2012). Nondestructive imaging techniques and analysis systems provide automatic phenotyping of root architecture (Iyer-Pascuzzi et al. 2010). Plant root monitoring platform (*PlaRoM* – software application) which includes noninvasive video image technique has been developed to examine root profiles in different genotypes (Yazdanbakhsh and Fisahn 2012). The other imaging and software platforms were improved for three-dimensional root observing for different traits in seedling stage (Clark et al. 2011). In this system, rotational image sequences are taken with optical system and 3D root architecture are generated by using the software, RootReader3D. High-throughput visual scoring technique permits fast selection of root structure especially in extreme areas (Trachsel et al. 2011). Semi-hydroponic phenotyping system with 240 L plastic mobile bins covered by black cloth and manageable watering system has been developed by Chen et al. (2011). Monitoring genotypes with this system was designed to host lots of plants in a small area and significant differences were observed for the traits of root length, branching, and density. X-ray computed tomography is a new nondestructive technique which provides detailed 3D root structure in smooth soils (Tracy et al. 2010). The other system is X-ray micro-tomography providing important information about root growth and root and soil interactions (Gregory et al. 2003). Wheat and rapeseed were imaged with the X-ray micro-tomography system at different periodic times. High-quality 3D images were acquired in developmental processes of the root parts. According to Gregory et al. (2003), this imaging technology is cheap and provides repeated scanning of live root systems compared with other tomographic techniques. In situ root phenotyping system in rice and soybean was developed by Fang et al. (2009). 3D images of roots were captured using 3D laser scanner. Generated 3D root structure had meaningful similarity with biomass and phosphorous content in rice and soybean. This approach by Fang et al. (2009) has therefore been evaluated as a new model for assessment of plant root growth processes in different environmental conditions. Magnetic resonance imaging (MRI) with positron emission tomography (PET) was used to study root/root parts in different soil types, with regard to their architecture, and transportation of water and nutritional elements (Jahnke et al. 2009). This noninvasive analysis provides information about root structures and transportation processes.

2.6 Limitations

Plant phenomics with high-throughput, state-of-art technologies has a great scope to advance plant science, thanks to interdisciplinary networking. Soil researches (environment), crop analysis, data researches with modeling, and sensor technologies are integrated into field and laboratories. This interdisciplinary network brings in a great speed, accuracy, efficiency to breeding, and optimized timing to crop management enabling non-biased and faster assessment of traits (Post 2011). However, plant phenomics has some limitations and/or disadvantages such as quality of measurable data, data regeneration costs, data collection technology, and availability of algorithms. In addition, phenotyping techniques are dependent on some factors such as simulations, sensors, active mechanisms, and high-throughput and field-based platforms (Post 2011). Further, most of the traits in plant phenotyping have some handicaps; for example, canopy temperature is an essential trait due its use for identification of certain physiological factors such as stomatal conductance, transpiration rate, plant water status, water use, leaf area index, and crop yield. However, trait expression shows interaction with both developmental phase and time of the day (e.g., pre-heading and/or morning readings are usually lower due to lower incident of solar radiation and air temperature) (Pietragalla 2012). Furthermore, weak

signals from plants in phenotyping confine to correct assessment of phenological stages (Furbank and Tester 2011). Measurement techniques such as measurement angle during the photoperiod also negatively affect proper canopy temperature identification (Jones and Vaughan 2010). The trait of stomatal conductance is very important to exhibit transpiration and gas traffic, especially in leaves. Some difficulties may emerge in measurement time for gas activity. Because stomata are susceptible to external influences and stomatal conductance, these may indicate different reaction to different leaves. Chlorophyll fluorescence is useful to exhibit drought resistance and it is one of the most used traits in phenomics. Even though fluorescence parameters can be determined easily, prediction values may change during the photosynthesis. Especially, in estimation of the PSII operating efficiency, several inaccurate measurements occur due to changes of fluorescence (Furbank and Tester 2011). Chlorophyll content is also important trait to identify photosynthetic activity; however, it is highly affected by environmental conditions. Solar angle, time, and leaf surface status might be obstructive factors for measuring chlorophyll content. In addition, leaf position in plant and calibration errors in chlorophyll meter have negative effects to chlorophyll content measurement. Canopy light interception provides highly useful information about crop growth and productivity and for crop modeling (Rosati et al. 2001). However, its measurement in field conditions is not simple and mostly affected by environmental fluctuations. Carbon isotope discrimination is beneficial in estimation of water status and transpiration capability; however, phenotyping of this character is very complicated. Other disadvantages of carbon isotope discrimination are its cost and needs for data transformation.

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High-Precision Phenotyping Under Controlled Versus Natural Environments

3

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3.1 Introduction

Multiple abiotic stresses such as drought, heat, cold, frost, salinity, high light intensity, adversely affect the growth and yield of agriculturally important crops. These stresses are recurrent and are becoming more serious under the current scenario of climate change. In order to develop climate-resilient crops with improved yield using various conventional and genomic approaches, primary strategies have to be made in establishing fast, precise and easy phenotyping for assessing available germplasm for tolerance to various abiotic stresses. The underlying physiological, biochemical, and molecular mechanisms responsible for tolerance to the above abiotic stresses are well characterized, and many morphophysiological traits have been identified which impart tolerance to these abiotic stresses. These traits are either constitutive or adaptive. The focus has given in the past years for harnessing the potentiality of adaptive traits indirectly towards the development of abiotic stress-tolerant genotypes. Currently, genetic diversity for both type traits is available in exiting germplasm of diverse crop species. Therefore, it provides enormous opportunities for developing stress-tolerant cultivars. However, it is required effective phenotyping methods that are rapid and reliable to screen the large number of genotypes. These traits can be screened under both natural and controlled conditions. Under field conditions, traits are highly influenced by environmentals factors, therefore stability of the traits of interest is often difficult to correlate with stress tolerance or yield. To create such complex situation of field in controlled conditions is practically impossible. The traits of interest measured under controlled environment and have reasonably good repeatability across different years of study should be evaluated under complex environmental conditions of field for confirming the stability of the traits. It is also equally important to know the underlying reasons why important traits for stress tolerance and yield do not hold good for different crops or why crops are different types of stress tolerant mechanisms. For example, The water-use efficiency measured through carbon isotope discrimination is well correlated with yield in groundnut but has less relevance to chickpea in terms of grain yield. Although high-precision phenotyping can be done for many traits related to abiotic stresses under natural conditions, there are many other traits that are only screened under controlled conditions. Moreover, certain traits are essential to screen because they are positively associated

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with yield or tolerance to abiotic stress and their measurement can only be possible in controlled conditions. Because the environment plays an important role in the growth and development of crop plants, crop plants face two different conditions during the phenotyping of interested traits. As a result each environmental condition has its own limitations. In this chapter, the precision phenotyping of traits of agronomic importance under the controlled and natural environments is discussed.

3.2 Environment and Genotype × Environment Interactions

Environmental conditions in which plants are grown play a vital role in generating the quality of phenotyping data through experiments. Consequently, it increases the efficiency of breeding. However, field variation increases error variances, thereby masking important genetic variation for key traits and reducing repeatability, regardless of the cost and precision of a phenotyping platform (Masuka et al. 2012). Soil heterogeneity even in relatively uniform experimental sites always has a possibility to mask the genetic effects, when a larger number of genotypes are phenotyped using high-throughput phenotyping. In that case it is difficult to find an area with minimum soil heterogeneity. Therefore, information on field variability can be incorporated into field designs by avoiding areas of high spatial variation (Jones 2007). These issues that have been discussed in the chapter for the "noise" factors are held constant, whereas the factor of interest ("signal") is allowed to vary. Environmental variability inconsistently affects phenotypic observation over both space and time and must be accounted during the phenotyping of traits. In modern high-throughput platforms, the implementation of environmental characterization is essential to facilitate data interpretation, metadata analysis, and, in the case of drought phenotyping, understanding patterns of water availability (Masuka et al. 2012). For screening of drought-resistant genotypes, the knowledge of soil moisture availability is a must to ensure that the field environment and the type of drought imposed are representative of the target environment (Romer et al. 2011). The development of wireless sensor networks to characterize both climatic and soil moisture conditions should enable real-time monitoring of environmental conditions (Fig. 3.3).

The genetic makeup of crop plants determines the physiological and behavioral responses of individuals under different environmental conditions. For quantitative complex traits, same genotype can produce a wide range of phenotypes in different environments. These phenotypic variations are attributable to the effect of the environment on the expression and function of genes influencing the trait. Changes in the relative performance of genotypes across different environments are referred to as genotype × environment interactions (GEI). During the past many years, many phenotyping platforms are emerging internationally and are being utilized in the screening of complex traits under controlled environments. However, many times, some specific traits showing large genetic variation in controlled conditions have a changed expression in the field. This is especially true for the techniques which are still limited to laboratory (Staedler et al. 2013) or glasshouse (Shi et al. 2013). In such situations it is essential to study GEI in multiple environments for harnessing the potential of high-precision phenotyping tools. The recent advances in crop phenotyping technologies have started to overcome this limitation (White et al. 2012).

3.3 High-Precision Phenotyping in the Field Under Natural Conditions

Pot experiments under controlled environment often cannot be extrapolated in natural field environments. The moisture extraction pattern under field is rather slow as compared to limited soil volume in pot culture which may lead to faster depletion of moisture (Poorter et al. 2012). Therefore, field conditions have been

used to phenotype the genotypes for different traits. However, those traits are more reliable which are stable and less influenced by environmental factors. One of the important physiological traits is water-use efficiency. This can be measured using leaf sampling for carbon isotope discrimination. The photosynthesis, stay green, chlorophyll content through nondestructive SPAD value, thermal imaging of canopy, transpiration, canopy cooling by infrared thermometry, stomatal conductance and root depth and mass measured indirectly through stable oxygen isotope ^{18}O are the parameters directly or indectly determine the plant water status and normal functional ability of the plant under stress environment. Field-based evaluation may be more pertinent for those characters which involve a combination of multiple traits such as canopy cooling which could be due to high root, stomatal conductance, and hormonal regulation. In the case of screening for drought tolerance, yield performance and flowering are evaluated under irrigated and rainfed conditions (Fischer and Maurer 1978). In this screening technique, yield under drought plots (rainfed/rainout shelter) and the potential yield under irrigation are to be compared for each genotype. The genotypes which showed low yield under drought relative to its irrigated counterpart had higher drought susceptibility index (DSI). The lower the DSI, the greater is the drought tolerance of the line. In chickpea, several genotypes such as RSG 143-1, RSG 888, Phule G5, Vijay, and ICC 4958 have been identified as having lower DSI using the above phenotyping techniques. Other traits used in phenotyping of drought-tolerant genotypes are exclusively associated with productivity such as dry matter, harvest index, and water-use efficiency and other drought resistance mechanisms comprising morphophysiological traits conferring resistance to drought (Passioura 1977).

For drought tolerance, high-precision phenotyping is being done using different approaches. For example, it can be done in dugout plots which are designed to create a moisture gradient, and different genotypes can be grown across the slope having different moisture gradients with minimum moisture on the top and maximum at the bottom. Genotypes tolerant to drought are expected to grow better in terms of biomass and grain yield with minimum availability of moisture at the top. Phenotyping for drought can also be done in rainout shelter. An automatic movable rainout shelter prevents raindrops to reach the plot where the material has been grown. A set of diverse germplasm are grown under rainout shelter, and the same are concurrently grown under rainfed and irrigated fields for comparison. This facility has widened the scope to identify genotypes tolerant to extreme drought conditions based upon their performance under moisture stress conditions.

3.3.1 Stage of Phenotyping in Field Conditions

Stage of the crop plant for phenotyping of a trait is most important for establishing a positive correlation between the targeted trait and the grain yield. For example, genotypic differences in the leaf ABA concentration in field-grown maize were maximum at the time of flowering. Therefore, in this case, ABA quantification is important at this stage for establishing a perfect genetic difference and its relationship with stress tolerance (Landi et al. 1995). Another example is the use of infrared thermography for measuring canopy temperature. In this case, the most appropriate strategy is to measure the temperature differences between treatments under the conditions of high evapotranspiration demand because genotypes can be differentiated by their ability to avoid stress. An additional factor to be considered when measuring canopy temperature is the effect of leaf wilting, folding, or rolling under stress condition (Grant et al. 2007). For instance, plant canopy architecture will influence leaf temperature not only through the angle of leaves to the light source but also through the degree of self-shading in the canopy (Zheng et al. 2008). The influence of self-shading can be reduced to certain extent if the most suitable view angle is used (Grant et al. 2006). Similarly, phenotyping is often biased if tested genotypes differ considerably in flowering time and/or the time of maturity. In such cases, phenotyping all

genotypes on the same date will actually represent data from plants at different physiological stages, which could introduce significant bias in the interpretation of cause–effect relationships between traits and yield. A partial solution is to sow the accessions on two or three dates based on the maturity group information (e.g., early and late).

3.3.2 Traits for Phenotyping in the Field

Development of cultivars especially for abiotic stress conditions required phenotyping of different morphological and physiological traits. These traits can be screened in the field or laboratory condition, sometimes in both conditions, which have been summarized in Table 3.1.

3.3.2.1 Simple Traits for Visual Observation

There are many traits in crop plants which are easy to observe, do not require any specialized instruments to measure them, and have been scored traditionally by plant breeders visually in the field. Many of such traits have been found to be associated with tolerance to biotic and abiotic stresses and hence can be used indirectly to select the tolerant/resistant genotypes. These traits include flowering time, improved plant type, leaf senescence, etc. For example, flowering time is considered as the most critical factor to optimize adaptation and grain yield under water-limiting condition (Richards 2006). Positive associations between plasticity of yield and flowering time across different levels of water availability have been reported in different crops (Sadras et al. 2009). Hence, phenology per se, plasticity of phenological development is considered to be a distinct trait influencing crop adaptation (Pinto et al. 2010; Sabadin et al. 2012). Selection of genotypes with shorter time to flowering has been reported highly successful for escaping the drought conditions in annual crops (Subbarao et al. 1995). For improving drought resistance in maize, a valuable selection target of intermediate heritability is provided by the anthesis–silking interval (ASI), which is usually negatively correlated with grain yield under drought conditions (Monneveux and Ribaut 2006). ASI can be phenotyped quite easily and effectively under the right experimental conditions, and substantial breeding efforts have targeted this trait through conventional breeding (Chapman and Edmeades 1999).

Sometimes improved plant type may give higher yield under water-limiting environments, although it may not have specific traits for drought tolerance. Thus, primary field level phenotyping for drought tolerance could be a simple function of genotype ability to perform well under water-limiting environments. The several interlinked traits that are considered as indirect estimates of photosynthetic potential are chlorophyll concentration, stay green, and delayed senescence (Shukla et al. 2004). Delayed leaf senescence maintains transpiration and increases cumulative photosynthesis over the crop life cycle (Vadez et al. 2011). Stay-green traits in maize correlate closely to grain yield, and multiple intervals of stay-green QTLs overlap with yield QTLs (Zheng et al. 2009). In sorghum it has been related to the maintenance of a more favorable water status as related to root features (Mace et al. 2012). In sorghum, four major QTLs that control stay green and grain yield have been identified (Harris et al. 2007), and near isogenic lines (NILs) for these QTLs have been derived, providing an opportunity for a detailed analysis of stay-green physiology and positional cloning of the underlying genes (Vadez et al. 2011).

3.3.2.2 Instrument-Based High-Precision Phenotyping

For high-precision phenotyping, several instruments have been developed that can be used directly to measure a trait in the field. These traits are early vigor, stomatal mechanism and transpiration, osmotic adjustment, leaf water potential, canopy temperature, plant and canopy features, etc. (see Chap. 2 for details). The leaf area index (LAI) and early biomass accumulation are important parameters to determine early growth vigor in crop plants. Leaf area meter can be used to measure leaf area of any geometry in laboratory followed by measurement of leaf

Table 3.1 Traits for phenotyping under field and laboratory conditions

Traits	Condition	Phenotyping requirements	Magnitude of labor	Remarks
Drought/heat susceptibility index	Field	Moisture gradient dugout plot/ rainout shelter/hot spots	Simple/slow	Yield variation can be explained under stress
Phenology	Field	Experimental field	Simple/rapid	Yield variation can be explained under stress
Root vigor/mass/ depth/ architecture	Field core sampling/ lysimeter/PVC tube/ pot culture/controlled greenhouse	Low-cost structure/facilities involved	Cumbersome	Yield variation can be explained under stress
Water-use efficiency	Controlled/field conditions	Pots for gravimetric determination/carbon isotope discrimination (Δ) through isotope mass spectrometer	Cumbersome for gravimetric and expensive for Δ. Rapid and precise	Yield variation can be explained under stress
ABA accumulation	Controlled environment	Immuno-linked radioimmunoassay	Tedious/slow	Yield variation can be explained under stress
Canopy temperature depression	Field condition	Infrared thermometer	Rapid/needs cautious attention	Yield variation can be explained under stress
Stay green	Field condition	SPAD chlorophyll meter/visual observation/pigment analysis	Simple and feasible/ rapid	Yield variation debatable under stress
Photosynthesis/ stomatal conductance	Field condition	Infrared gas analyzer	Tedious/slow/ variable	Yield variation debatable under stress
Leaf area index/ leaf orientation/ leaf reflectance	Field condition	Canopy analyzer	Feasible/ cumbersome/slow	Yield variation can be explained under stress
Thermal imaging	Field condition	Thermal image analyzer	Slow	Yield variation can be explained under stress
Specific leaf area	Field cum laboratory	Weighing balance/leaf area meter	Slow	Yield variation debatable under stress
Leaf waxiness	Field cum laboratory	Chemical analysis	Slow	Yield variation debatable under stress
Osmotic adjustment	Field cum laboratory	Osmometer	Cumbersome	Yield variation debatable under stress
Membrane stability	Field cum laboratory	Conductivity meter	Cumbersome	Yield variation debatable under stress
Proline accumulation	Field cum laboratory	Chemical analysis	Cumbersome	Yield variation debatable under stress
Lethal leaf water potential	Field cum laboratory	Pressure chamber	Cumbersome	Yield variation debatable under stress

(continued)

Table 3.1 (continued)

Traits	Condition	Phenotyping requirements	Magnitude of labor	Remarks
Relative water content/leaf water potential	Field cum laboratory	Pressure chamber	Cumbersome	Yield variation can be explained under stress
Pollen fertility	Field cum laboratory	Specific stains and microscope	Moderately fast	Yield variation can be explained under stress
Remobilization of water-soluble carbohydrates	Controlled environment	Radio tracer/mass spectrometer/ scintillation counter	Slow and time effective	Yield variation can be explained under stress
Chlorophyll fluorescence imaging and allied parameters	Field cum laboratory condition	Fluorescence imaging system/ fluorometer	Slow and precise	Yield variation can be explained under stress
Antioxidant enzyme activities	Laboratory condition	Biochemical analysis	Slow and variable	Yield variation debatable stress

area index (LAI) using canopy analyzer under field conditions. This can precisely monitor LAI (total canopy area covered per unit ground coverage) during progressive growth of the crop. It has been shown that higher initial LAI correlates positively with grain yield under water-limiting environments. Thus, this trait can be easily phenotyped under field conditions. The early extensive biomass accumulation and setting of reproductive organs before onset of terminal drought has been important for identifying the genotypes escaping drought. Early vigor under low evapotranspiration conditions helps to optimize water-use efficiency (WUE) and limits water loss due to direct evaporation from the soil surface. As a result, more stored water is available for later developmental stages when soil moisture becomes depleted to a great extent and limits yield (Rebetzke et al. 2007; Richards et al. 2007). The reduction in the stomatal density and an increase in the leaf reflectance through production of glandular hairs in chickpea are considered to be adaptive traits through which leaves reduced water loss and intercepted non-photosynthetic radiation. Isotope ratio mass spectrometer (IRMS) in leaf samples is very simple and can be applied for large-scale screening of crops for higher water-use efficiency with less time and more precisely under field condition. Tissue samples in large numbers may be harvested from the field and oven dried and analyzed through IRMS (isotope ratio mass spectrometer).

The loss of water through stomatal aperture called transpiration is severely affected when crop is subjected to water stress. The transpiration rate can be measured nondestructively by using infrared gas analyzer or porometer. Osmotic adjustment (OA) is considered as an important physiological trait for adaptation to drought. The OA in chickpea has been reported to range from 0 to 1.3 MPa. The OA increases water absorption; maintains cell turgor, photosynthesis, and leaf area duration; helps stomatal opening; delays senescence and death; reduces flower abortion; and improves root growth as water deficit develops (Basu et al. 2007b). The greater osmotic adjustment leads to higher growth rate and dry matter production under drought. The degree of OA has also been shown to be correlated with yield under dryland conditions in chickpea (Basu et al. 2007a). Leaf water potential can be measured either by using thermocouple psychrometer or pressure chamber. The detached leaf is sealed in a steel chamber with only the cut end (petiole) protruding out. Pressure is applied to the chamber (from a pressure source such as a compressed nitrogen

cylinder). When the sap meniscus appears at the xylem surface, the pressure is recorded and taken as the xylem (leaf water) potential. Normally −0.2 to −0.5 MPa value of the leaf tissue is considered as that of a non-stressed leaves having almost full turgidity, although crop species show different LWP at full turgidity. If the LWP of a crop species declines below −0.5 MPa, it is a sign of stress which increases with water deficit.

Infrared gas analyzer or chlorophyll fluorescence by pulse amplified fluorometer provides a nondestructive probe of photosynthetic activity. It has been used to investigate effects of stress on photosynthesis. Canopy temperature is related to the amount of dehydration occurred during the growth of development of plant canopy under the stress conditions, and hence, it is an important selection criterion to identify high-yielding genotypes under drought (Olivares-Villegas et al. 2007). It can be measured using infrared thermometry in the field, which is a simple instrument for rapid indirect phenotyping for drought tolerance. For precise measurement of the plant features and crop growth, digital image analysis and video image analysis are cheap and rapid and nondestructive methods (Fiorani et al. 2012; White et al. 2012). Remote sensing through spectral reflectance and near-infrared spectroscopy of crop canopy-based sensors are being utilized in high-throughput phenotyping platforms for collecting integrated traits with high temporal resolution (Montes et al. 2007; Gutierrez et al. 2010). Remote sensing has advanced our understanding of changes in leaf reflectance and leaf remittance according to species, leaf thickness, canopy shape, leaf age, nutrient status, and, more importantly, water status (Hatfield et al. 2008). Using these informations, various vegetative indices for crop canopies have been formulated in order to quantify agronomic parameters such as leaf area, crop cover, biomass, yield, etc. The spectral reflectance is a powerful tool that has been used to monitor plant photosynthetic pigment composition, water status assessment, and the early detection of abiotic stress in field conditions (Gray et al. 2010). Recently, a high-throughput technique known as "shovelomics" has been deployed to investigate several root architectural features in field-grown maize (Trachsel et al. 2011).

3.3.2.3 High-Precision Phenotyping Based on Laboratory Techniques

Advances in high-throughput phenotyping have led to development of several laboratory-based tools and techniques which have been used to screen physiological traits, especially those related to abiotic stresses. These techniques use plant samples including leaf and other parts grown in the field. The traits include stomatal conductance, water-use efficiency, osmotic adjustment, abscisic acid concentration, chlorophyll content, pollen germination, etc. (see Chap. 2 for details). The stomatal conductance helps to regulate the water balance of the plant. It can be measured on basis of $\Delta^{13}C$ in laboratory and has been used successfully to identify a number of bread wheat cultivars released by CIMMYT from 1962 to 1988 that showed a strong positive correlation between stomatal conductance and grain yield ($r = 0.94$; Fischer et al. 1998). However, fluctuating environmental conditions (wind, solar radiation, humidity, etc.) during the day actually affect accurate measurement of stomatal conductance of a reasonably large number of plants. It can be measured using natural oxygen isotope composition ($d^{18}O$) in leaf and grain materials for large number of samples with very little labor in the field (Ferrio et al. 2007).

Water-use efficiency (WUE) is the main limiting factor to crop production. Therefore, it is an important physiological trait for screening drought-tolerant plants growing in the field. It can be estimated precisely in laboratory by using carbon isotope discrimination technique and has already been used to identify the genotypic differences among genotypes of groundnut and chickpea lines (Kashiwagi et al. 2006). The concentration of ABA in the leaf tissue and guard cells of stomata influences leaf temperature via an effect on transpiration through stomatal

conductance (Wasilewska et al. 2008). Therefore, ABA plays major role in regulating the water demand and supply and to optimize growth and survival in response to short-and long-term environmental fluctuations (Xiong et al. 2007). It has been used as a trait to identify genotype tolerance to drought and other abiotic stresses (Setter 2006) in several crops including maize (Sharp et al. 2004; Hose et al. 2001), pearl millet (Kholova et al. 2010a, b), and chickpea (Zaman-Allah et al. 2011a, b). In cereals, an accumulation of ABA influences reproductive fertility (Zhang et al. 2009) and endosperm development (Seiler et al. 2011). Genetic variability for sensitivity to ABA has been reported in maize also (Frascaroli and Tuberosa 1993).

Heat stress at reproductive stage has become a serious constraint for many crops due to climate change, and the critical temperature ranging from 35 to 40 °C damages reproductive organs leading to drastic yield losses. It has been shown that pollen germination and the degree of pollen tube growth are reduced significantly at high temperatures (Kakani et al. 2005). However, sensitivity for higher temperature has been shown to be variable among genotypes. Therefore, pollen viability in respect to heat stress has formed a strong basis of phenotyping for identifying genotypes having tolerance to high temperature. It is very simple to detect pollen viability from the unopened flowers collected from field-grown crops by staining with acetocarmine solution or 1 % TTC (Triphenyl-tetrazolium chloride) solution. The viable pollens will take deep stain, while sterile, nonviable pollen grains will remain unstained. However, for testing absolute fertility, i.e., in vivo pollen germination on stigma, heat-treated flowers are collected and processed with Alexander's stain to validate pollen tube growth on stigma tips (Pratap et al. 2014). In chickpea, tolerant chickpea genotype ICCV 92944 showed maximum percent pollen germination at critical 35 °C temperature (Fig. 3.1). Similarly, Pratap et al. (2014) identified one accession each of *V. umbellata* and *V. glabrescens* insensitive to higher temperatures.

3.4 High-Precision Phenotyping in Controlled Conditions

Development of improved genotypes through breeding requires phenotyping of a large number of genetic resources. Phenotyping for secondary morphological traits such as plant height, leaf number, flowering date, and leaf senescence is simple and easy in the field. However, traits associated with stresses are required to be phenotyped under controlled conditions for a better understanding of the stresses (Weber et al. 2012). Therefore, it requires managed environment facilities for increasing the accuracy in measurement of traits (Rebetzke et al. 2013; Blum 2011a). There are many traits which can only be screened in controlled conditions (Table 3.1). For example, photosynthetic efficiency can be rapidly and accurately measured under laboratory controlled environment using chlorophyll fluorescence imaging system. Many of the traits like roots mass/vigor, high membrane stability, high photosynthetic efficiency, etc. are commonly addressed to multiple stress tolerance such as drought, heat, cold, and salinity. Hence, phenotyping of these traits should be done with utmost accuracy in either condition.

For root-based traits, phenotyping is difficult in field conditions and hence requires controlled lysimetric facility/rhizotron and rainout shelter or greenhouse for screening large number of germplasm. Since stress tolerance is a complex metabolic and regulatory functions, many methods have been developed for estimation of such traits in controlled environment. But some of them are too time-consuming, cumbersome, expensive, or requires high technical efficiency for large-scale phenotyping. Controlled conditions are required to those traits, which are highly influenced by environmental factors in the field. For example, plant growth is highly variable under field conditions, and hence, natural conditions are required to simulate in the controlled conditions for measurements of target traits related to plant growth (Izanloo et al. 2008). The controlled environment facilities such as greenhouse, growth chamber,

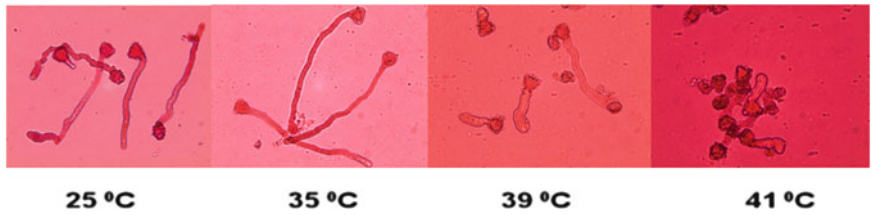

Fig. 3.1 Pollen germination in chickpea in response to high temperatures

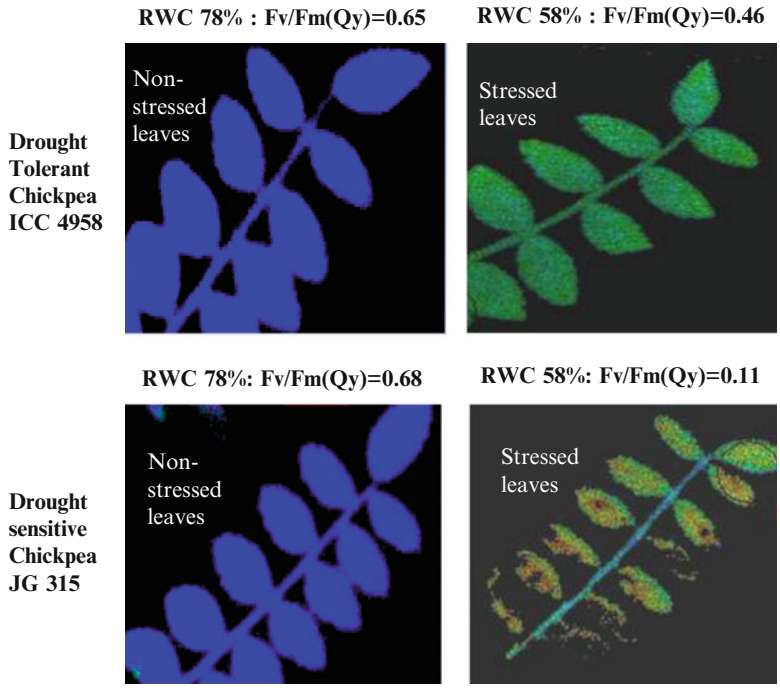

Fig. 3.2 Image of quantum yield indicating irreversible damage of photosynthetic system in leaf subjected to stress (RWC 58 %) in comparison to non-stressed condition (RWC 78 %)

etc. control the major environmental parameters like temperature, light, drought, and relative humidity leading to precise monitoring of traits of interest. For example, exposing test plants to a solution with a known concentration of polyethylene glycol (PEG; molecular weight >6,000) with good aeration of the solution to avoid hypoxia provides uniform conditions in terms of water stress. Thus, exposing different genotypes to a given level of dehydration under similar environmentally controlled conditions allows phenotyping of germplasm for drought tolerance more precisely unlike field conditions where they are likely to experience different stress intensities (Ruta et al. 2010). Other stress-adaptive traits such as osmotic adjustment, membrane stability, pollen fertility and remobilization of carbohydrates, expression of antioxidant enzyme complex and chlorophyll fluorescence, etc. are required in controlled environment for precise phenotyping. Fluorescence imaging system has been used to assess response to photosynthesis among genotypes of chickpea. Effects of stress modify photosynthesis, which can be precisely monitored through change in the chlorophyll fluorescence patterns leading to change in Fv/Fm (Fig. 3.2). Light response of ETR (electron transport rate) as a measure of proxy photosynthesis showed high ETR values in drought-tolerant chickpea genotypes under water stress at

Fig. 3.3 Screening for root traits in PVC tubes (*Left* IIPR, Kanpur Research station and *Right* Lysimeter facility at ICRISAT, Patancheru, India)

high irradiance levels as compared to the sensitive lines (Fig. 3.2). Thus, fluorescence imaging and allied parameters allow high-precision phenotyping under controlled environmental conditions.

Environmental factors markedly influence transpiration efficiency (TE) as measured through gas exchange system under field condition; therefore, it is difficult to assess real genetic variation in TE through gas exchange. However, measuring gas exchange parameters and CER/gs (Carbon dioxide exhange rate/Stomatal conductance) in controlled conditions may considerably limit the effect of environmental variation (Poorter and Farquhar 1994; Kalapos et al. 1996; Fischer et al. 1998).

Root traits are important traits that can be screened in controlled environmental conditions. Root scanner with Delta-T root scanning software is used to measure total root length, perimeter, thickness, and total surface area. However, few systems have been developed to screen genotypes with higher root profile using 18 cm diameter polypropylene PVC tubes (Fig. 3.3). In such experiments, the soil in tubes was tightly packed, and compactness almost similar to the field condition was maintained. The soil inside the tubes was irrigated at first instance to its field capacity level, and thereafter sprinkler irrigation was applied. When the plants reached a stage of 25–30 days, tubes were taken out and were cut longitudinally. The soil surrounding the roots was washed out with mild flow of water to extract intact roots. This system was used to screen a large number of chickpea genotypes, for example, ICCV 92944, ICC 4958, BG 256, Phule G 5, ICCV 94916, and RSG 143-1 which were identified as ideal donors for high root mass.

Canopy temperature depression measured by thermal imaging is the difference in temperature between the canopy surface and the surrounding air. It is a highly integrating trait involving several biochemical and morphophysiological features acting at the root, stomata, leaf, and canopy levels. Higher canopy temperature depression under field conditions is related with a cooler canopy temperature under drought stress, and genotypes having higher canopy temperature depression use more of the available water in the soil to avoid excessive dehydration (Reynolds et al. 2009). Infrared thermometry data is used to measure close differences in leaf temperature in both field and controlled conditions (Winterhalter et al. 2011). Thermal imaging measures this trait indirectly and used for detecting changes in stomatal conductance and leaf water status in a range of plant species (grapevine, bean, and lupin) under greenhouse or controlled environment conditions. The absolute leaf temperature has been found to be related with thermal indices of plant stress, stomatal conductance, and water potential.

3.5 Limitation of Using High-Precision Tools in Controlled and Natural Conditions

High-throughput techniques noninvasively capture information throughout the plant life cycle in

a carefully controlled environment, which are used to identify quantitative trait loci and candidate genes. However, genes identified within controlled environments are generally not translated into gains in grain yield in the field (Araus et al. 2008; White et al. 2012; Von Mogel 2013). This is because of the notorious heterogeneity of field conditions and our inability to precisely control environmental factors. This makes results difficult to interpret. The controlled environments are far removed from the situation that plants will experience in the field and, therefore, are difficult to extrapolate to the field (Blum 2011a, b; Passioura and Angus 2010; Passioura 2012). The following are the limitations which are associated with high-precision phenotyping under the controlled conditions:

- The volume of soil used within a pot is considerably smaller as compared to the field. Consequently, it limits the availability of water and nutrients to plants (Passioura 2006; Poorter et al. 2012; Reynolds et al. 2012).
- The soil environment, which plays a crucial role in plant growth and development, is difficult to simulate in controlled conditions (Whitmore and Whalley 2009).
- Screening against drought stress is challenging because declining soil moisture content is associated with increased mechanical impedance in the field, which is an effect that is difficult to replicate within pots (Cairns et al. 2011).
- Traditionally, genetic improvement for varietal development commonly depends upon multilocation screening within the target environment (Cairns et al. 2013). Consequently, plants experience a range of stresses or environmental variations throughout their life cycle. Usually, in many cases, the environmental characteristics are not monitored and, hence, are poorly understood. In those situations, it becomes difficult to control such environmental conditions under controlled environments.

The recent developments in wide array of advanced tools and techniques have led to development of several high-throughput phenotyping tools for different morphological–physiological traits under the field conditions. Although these tools are now allowing phenotyping of a number of traits, implementation is still slow due to the following reasons (Cobb et al. 2013; Costa et al. 2013):

- The phenomic platforms use the advanced phenotyping tools and techniques to generate the volumes of phenotypic data and their management and analysis becomes difficult (Maes and Steppe 2012; Pieruschka and Poorter 2012). Therefore, advanced analysis tools are required beyond even the usual statistical tools (White et al. 2012).
- Much of the data generated in high-throughput phenotyping platform are just mathematical transformations of numbers, and hence, it is difficult to understand them (White et al. 2012). We do not even have a physical concept of what some of the numbers obtained by high-throughput phenotyping platforms mean in terms of plant or crop performance (Cobb et al. 2013).
- Pyramiding all levels of information (different categories of traits measured at different times, spatial variability, environmental information) in a coherent manner requires the setting down of a strong modeling foundation based on a wide but deep understanding of the ecophysiological and genetic factors determining crop performance.
- More user-friendly post-processing of the raw data generated is needed. Improved software tools to optimize automation and speed up robust data analysis should support such a trend (Fuentes et al. 2012).

3.6 Conclusion

Phenotyping under field conditions still remains a bottleneck for future breeding advances (Cabrera-Bosquet et al. 2012; Cobb et al. 2013; Araus et al. 2008; Cairns et al. 2012a, b). Besides the above considerations, the choice of phenotyping under controlled conditions versus field environments largely depends on the purpose of phenotyping and the heritability of the

trait, together with the logistical considerations of collecting the data (Cobb et al. 2013). For example, there are no feasible spatial or temporal options for testing high atmospheric CO_2 in the field (Gleadow et al. 2013). However, efforts are being made to manage the same environmental conditions of field under controlled conditions. Few platforms have been developed for studying the genotype and environment interactions. However, focus is required to develop the models and bioinformatics facility to analyze huge amount of phenotypic data generated through high-throughput phenotyping tools and techniques and their easy understanding. In recent years, rapid progress has been made in the development of a wide array of technologies including novel sensors, image analysis and modeling, robotics, and remote control and data mining. These tools now allow phenotyping of a number of traits faster, i.e., within seconds under field conditions. Use of these developments is required on large scale under both field and controlled conditions as per the requirement.

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Toward Digital and Image-Based Phenotyping

4

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Abstract

Manually performed measurements in field phenotyping are labor- and time-consuming, often destructive and not objective. Moreover, the complexity and variability of crop plants under field conditions require high-resolution data and filtering. As a consequence, there is a need for spatially and temporally differentiated data, objective data acquisition, and high-throughput technologies. Image-based systems, selective on morphological and spectral crop characteristics, are adequate sensors for further interpretation of raw data in terms of crop properties. In particular multi-sensor and data fusion has a potential to compensate the varying influences of sunlight, dust, moisture, or uneven land in the field. Due to the high-resolution data of image-based systems – such as digital color cameras, spectral imaging, laser scanning devices, or 3D cameras – detailed crop properties have become available, even individual plant phenotyping is an option. Autonomous field robots have a high potential for field phenotyping as well as new sensor technologies and virtual phenotyping. Data management is of relevance for field phenotyping, starting from storing the large amounts of raw data up to artificial intelligence algorithms for trait determination. Interdisciplinary cooperation is crucial for the implementation of digital phenotyping into practice.

Keywords

Digital phenotyping • Image-based sensors • Sensor fusion • Virtual phenotyping • Field robot

4.1 Introduction

The increasing world population related with growing demands for food and energy, limited resources, environmental impacts from agricultural processing, or climate changes has

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generated new challenges in crop farming. Since the number of influencing parameters on most of the agricultural and environmental processes is very high and not completely understood, there is a strong need for increasing the knowledge in order to find sustainable as well as practical solutions. This is in particular true for plant breeding, where genomic approaches have been improved significantly while phenomics in crop fields has changed little in the last decades. The resulting lack of phenotyping information together with comparably low-cost genomics has resulted in the recent focus on plant phenomics. However, the complexity for plant phenome analysis is very high due to the large variability of interactions between the environmental factors (such as soil properties or weather) and agricultural operations (such as fertilization or crop protection) with the genome of the plants. Moreover, dynamic effects of these parameters and others (such as crop rotation) are also of importance. As compared to this complexity, the state-of-the-art phenotyping does not supply sufficient information. Moreover, most of the manually performed measurements are labor- and time-consuming, often destructive and not objective (Montes et al. 2007). As a consequence there is a need for much more spatially and temporally differentiated data, objective data acquisition, and high-throughput due to the large number of field plots to be evaluated. Consequently, information and communication technologies are key components to solve the bottleneck in plant phenotyping (Fiorani and Schurr 2013).

Developments in electronics, robotics, computer science, and sensor systems have already offered new options in agriculture during the last 25 years (Mulla 2013). This tendency is summarized as "precision farming" (Auernhammer 2001). Technologies such as GPS, sensors, embedded and communication systems, human-machine interfaces, and mechatronic systems are dominating innovations in agriculture. All these technologies are also needed for crop phenotyping; thus in particular, the application of sensors in agricultural environments is not new. For agricultural applications, a deterministic ("online") data handling is of utmost importance. Examples are the usage of online sensor data for direct fertilizer control (Link and Reusch 2006) or the online detection of crop properties for adjusting the cutting height during maize harvesting (Egbers et al. 2006). The corresponding data have to be collected, interpreted, and used for open- or closed-loop control interaction for online applications. As compared to the sensor application for agricultural processes, although the demands for crop phenotyping have similarities, the focus is different with respect to the following aspect. The data are not used for the control of mechanical systems; thus, there is no need for a real-time system, excluding the storage of the data. Moreover, for phenotyping, the requirements with respect to the spatial resolution of measurements are typically higher.

It is interesting to notice that a kind of "phenotyping systems" has already been developed for other agricultural processes several years ago. These systems have used imaging systems for crop-weed detection in order to apply mechanical weeding or chemical weeding with a reduced amount of herbicides (Zhang and Chaisattapagon 1995; Gerhards et al. 1998). Since the crop-weed detection or identification on the go needs data with a high spatial and temporal resolution, image-based systems – such as a cameras or light curtains – as well as sensor fusion concepts have already been used in 1998 (Ruckelshausen et al. 1999). For typical crop phenotyping applications, the demands are similar to the weed control examples; thus, the amount of raw data is typically higher as compared to state-of-the art agricultural processes, for example, sensor-based fertilization.

The key components of digital phenotyping for nonsubjective measures (Darrigues et al. 2008) thus include the real-time data storage with a high spatial and temporal resolution and a subsequent (offline) data analysis. Due to these boundary conditions and the expected quality, imaging systems are in focus for phenotyping as, for example, distinguishing between crop and soil is extremely complex or even impossible without imaging data. The offline data analysis

includes models and calibrations to extract crop and trait parameters from the raw data, while sensor data itself do not give direct information about the crop status.

The development of the technologies can be performed – similar to industrial applications – in the laboratory or in greenhouse environments. However, the phenotyping for crop plants has to be performed under agricultural application conditions, on the field. Moreover, due to strong variations of soil, crop, weed, and environmental conditions, typical industrial systems, such as machine vision technology, cannot be directly applied for outdoor applications. The influence of sunlight, wind, dust, humidity, rain, or vibrations is a challenge for sensor systems in agriculture; thus, the availability of commercial sensors or imaging systems for crop analysis in agriculture is very limited. Considering both aspects, the complexity of crop property measurements on the field itself and the challenges of imaging under outdoor conditions, this chapter focuses on digital and image-based systems for field phenotyping. Next to the data acquisition and analysis, application-oriented aspects of digital field phenotyping have to be added, in particular, in the physical system for hardware integration, a carrier for positioning the system in or above the field. The major aspects, options, and limitations of digital phenotyping are described in this chapter, including image-based systems (Sect. 4.2), data management (Sect. 4.3), and the phenotyping process in crop fields (Sect. 4.4). Future trends are given in Sect. 4.5.

4.2 Image-Based Systems for Crop Phenotyping

Technology and equipments are applied to measure crop or environmental properties in manual phenotyping. For nondestructive investigations, height measures or spectrometers are the typical tools. In the case of nonimaging devices (such us spectrometers for NDVI measurements), the selection of the material is done by the user, and typical examples are selected on the basis of human experience and image selection. Thus, the raw material for phenotyping is based on a subjective selection, a kind of human image processing. Even when a camera is used, the selected image of field view depends on the user. As a consequence, there is a need for objective measurements based on sensor systems and its corresponding analysis. However, for a fully technological solution, there is the challenge to find representative images – previously selected by the user – automatically. Due to the fast increasing progress in image processing technologies (Davies 2012), as a cross-section discipline, digital phenotyping can adapt existing algorithms to the specific application, in particular to the varying conditions of crop plants and environmental conditions (see Sects. 4.3 and 4.4).

The idea of applying imaging to agriculture goes along with the technological progress of image capturing and processing technologies. Long before integrated circuit CCD or CMOS image sensors became available in the 1980s, while remote sensing for agricultural applications was already a research topic in the 1960s (Landgrebe and Phillips 1967) and in the 1970s (Haralick 1976). The availability of digital imaging had a strong impact to the exponentially growing number of applications, including machine vision technologies in agriculture (Chen et al. 2002). However, imaging under dynamic varying measurement conditions in an outdoor field (Hellebrand et al. 2002) is much more complex as compared to quasi-static conditions for indoor industrial production processes. As a consequence, first commercially available products of digital image processing have become available for defined environmental conditions, such as greenhouses, indoor production lines, or capsulated devices on machines, for example, the inspection of cereal grains (Davies 2012). Under outdoor conditions, driver assistance systems have become available in the past years based on laser scanning or digital cameras; however, the detection of crop properties under outdoor conditions is still a challenge.

In contrast to driver assistance applications, non-destroyable sensors for crop analysis under

agricultural field conditions are still nonimaging devices. Most of these commercially available sensors are used for detecting green structures on soil or measuring spectral crop properties for fertilizer control (Oerke et al. 2010 and references therein). However, the complexity and interpretation of the agricultural processes are very high, such as the technical complexity of the fertilizer technology itself. As mentioned above, the varying influence of environmental and other parameters strongly effects the development of robust systems. For example, the interpretation of nonimaging sensor information of an area partly covered by crops and soil is very hard or even impossible to solve, the same is true for varying environmental impacts. For optical measurements as, for example, changing sunlight intensities have to be taken into account. For this, uses of active light sources are most robust with shading devices or at night from a sensor point of view measurement.

The nonimaging devices available in the market do not fulfill the typical quality requirements for digital phenotyping. The spatial resolution implies the usage of images in the very first phenotyping step of data collection on the field. The sensors for raw data capturing are thus key components for digital phenotyping. While on a first glance classical color cameras are in focus, there are several options to obtain an image with other technologies, for example, images at selected spectral ranges or 3D distance information. It is even possible to generate an image out of individual point-wise sensor data, such as a light curtain image sensor (Ruckelshausen et al. 1999; Fender et al. 2005). Thus, in this chapter, the terms image-based sensors and image-based phenotyping will be used. Moreover, image is used in an abstraction where for each coordinate pair (x, y), an intensity information is given. Coordinates could be the spatial position or the wavelength. Examples for the intensity are the distance or the light intensity. The resolution of the intensity information is ranging from 1 bit (binary image) up to commercially available 16-bit camera systems. The number of coordinate pairs – for cameras, these are picture elements ("pixels") – can vary from a few hundred up to several millions.

Moreover, an integrated vision system is affected by a large number of influences, ranging from technological parameters via application-specific aspects up to costs (Davies 2012). Motion and real-time data acquisitions – better: a deterministic behavior – are of high relevance for dynamic field measurements where the loss of information is not acceptable and can result in the misinterpretation of plant breeding processes. In this section, we will focus on the contributions of digital imaging to digital phenotyping, while in Chap. 5 further information is given about specific aspects of imaging methods for trait determination.

For understanding the options and restrictions of image-based sensors for digital phenotyping in the field, there is a need for looking at the specific basic functionality, selectivity, and possible disturbance variables of the different devices. The categories of image capturing can be structured in several ways; examples are ranging from physical parameters (such as reflection or travel time measurement) up to the final interpreted or even modeled data (such as plant height, moisture content, or biomass). However, it is of high relevance to clearly distinguish between the image capturing process with raw data as a result and the final model-based phenotyping result with crop properties or trait determination (as described in Sect. 4.4). Using the same raw data, different models will typically lead to variations in crop properties as the final result. Sensors are categorized with respect to the image capturing process, while the phenotyping process (Sect. 4.4) results in crop characteristics. Major measurement signals for field phenotyping applications refer to spectral and morphological signals, while other image-based methods are usable for indoor phenotyping and have still been developed further for being applied under outdoor conditions.

4.2.1 Spectral Selectivity

Classical cameras are based on silicon sensors and can thus detect light in the visible (VIS) and near-infrared (NIR) range up to about 1,100 nm. Depending, optical filtering cameras can be designed as gray value systems (without filter and thus a higher spectral sensitivity) or color cameras (with typically red, green, and blue filters) for further color analysis. Both types are integrating a broad range of wavelengths (in the order of 100 nm or more) in one or three individual output channels. Digital color cameras have broadly been applied in research for measuring crop properties (e.g., Pan et al. 2007 or Li et al. 2010). By using several image sensors with optical splitting, so-called multispectral cameras can supply one or more additional NIR channels for advanced image processing with higher selectivity and higher spatial resolution.

Hyperspectral imaging can be applied if a higher spectral resolution is needed (for details, see Chap. 6). While for static applications mechanically rotated or programmable optical filters can be used, outdoor phenotyping applications typically apply line sensors where for each position a complete optical spectrum is supplied. By storing the two-dimensional position, wavelength-matrix point-wise spectral analysis can be performed (Mulla 2013 and references therein). The continuous spatial image in dynamic measurements is generated by integrating line by line, thereby resulting in a three-dimensional cube (x and y position versus wavelength).

4.2.2 Morphological Selectivity

While the image-based systems described above are reducing the information from a three-dimensional world to a two-dimensional plane, the 3D information is of high interest for analyzing distances, geometric or morphological structures. In the recent years, different technologies have been developed and are available as commercial products. Laser distance measurements have already been applied for crop plant measurements; 2D- and 3D-laser scanning devices are used in agriculture for a few years and support point-wise distance information for further image processing (Preckwinkel et al. 2004; Gebbers et al. 2011; Claas 2013). By using a laser line as active light source, image-based distance information can be determined by triangulation. Such systems based on the laser light sheet method are already available for crop phenotyping (Lemnatech 2013). The impact of varying sunlight conditions is still a challenge for such systems, which is also true for 3D time-of-flight cameras, which have become available in the last few years (Klose et al. 2012). There is also a high potential in phenotyping for low-cost consumer imaging systems such as the Kinect camera (Fossati et al. 2013); however, the disturbances of the projected light pattern by sunlight still hinder field phenotyping applications. An interesting image-based technology for phenotyping is the measurement of a two-dimensional shadow of crop plants, which can be generated by placing optical sensors and emitters opposite to each other with the plants in between. For this purpose, an individual laser line or an LED can be used as light source on one side, while CCD or CMOS image sensors (for high resolution) or individual photo diodes (for lower resolution) are placed on the other side. Such systems have already been applied in the field for crop-weed detection long ago (Ruckelshausen et al. 1999) but have come into focus for phenotyping applications more recently (Busemeyer et al. 2013a).

4.2.3 Other Selectivity

There are a large number of specific imaging systems which might come into focus for phenotyping applications in the future; however, their impact on state-of-the-art research and development in field phenotyping is still low. All sensor principles and technologies might be applied to digital field phenotyping; however, the complexity or the robustness of new technologies is not yet suitable for these applications. For

indoor or greenhouse phenotyping, some of the technologies have already been applied or are addressed in research projects.

Magnetic resonance images (MRI) are broadly used in medical applications and have a high selectivity, in particular with respect to the complete volume of an object as compared to VIS/NIR systems detecting the surface region. These systems are applied for to plant root analysis (Schulz et al. 2013) and even have the potential for mobile imaging applications, such as analyzing the growing process of pear fruit (Geya et al. 2013; Windt and Blümler 2013). The multiple reflection of an individual ultrasonic pulse by crop plants and soil can be interpreted in terms of crop levels, and first field experiences are available (Reusch 2009; Makeen et al. 2012).

During the past few years, significant progress has been made in developing terahertz technologies for material analysis. The first applications on measuring crop properties, especially the leaf water content, have been performed (Breitenstein et al. 2011; Gente et al. 2013). Applying radar for crop detection has always been a topic for research, including the extraction of features for crop classification (Anys and He 1995). The application of wideband radar is also a selective option for detection of crops or plant roots within the soil (see e.g., Konstantinovic et al. 2007). X-ray technologies (such as computer tomography) have already been applied for indoor crop root analysis and showed future potential (Fiorani and Schurr 2013). For top soil mapping, passive γ-ray measurements (van Egmond et al. 2010) have already reached a commercial product level (Medusa 2013).

4.3 Data Management

The collection and storage of phenotyping sensor data are still a challenge, no matter if digital or manual phenotyping is applied. In particular if data from different users are integrated or previous phenotyping data are combined with newer ones, detailed information about the measurement conditions and data formats are necessary to support an efficient data interpretation (Billiau et al. 2012). In digital phenotyping, several sensors are typically used for the collection of phenotyping information (such as described in Sect. 4.2) and its interpretation (as, e.g., GPS, plot data). Consequently, a systematic data acquisition is crucial from the beginning in order to supply the input data for crop property interpretation. Thus, on one hand, the handling of "big data" on the field makes the phenotyping process technologically complex; on the other hand, a systematic data management supports the generation of a transparent data structure for further processing and multiuser purposes.

The first step in the data management process of phenotyping platforms is the raw data collection and storage. Typically, each sensor can supply a data package within a given time; one data package can be named a frame (as typically used in imaging), and the corresponding data rate is correspondingly designated as frame rate. Frame sizes and frame rates typically vary strongly. For example, a GPS sensor supplies a few bytes in a second, while a hyperspectral imaging system supplies a few megabytes with frame rates up to a few hundred Hz or a laser distance sensor with a few bytes in the range of kHz. These data have to be stored in relation to the field position. The technological challenges strongly depend on the phenotyping application (Busemeyer 2013):

- If the interpretation of raw data is related to a larger area, such as a field trial plot of a few square meters, at a first view it seems that there is no need for a higher local accuracy as enabled by image-based systems. As an example we have a look at a nonimage-based spectrometer with a top-view measurement of a field plot including crop gaps. In this case, the spectrometer is detecting mixed spectra including crop as well as soil components. Thus, the average value does not represent crop properties. It is important to notice that this way of data collection can limit the data analysis or even lead to wrong phenotyping results. As a consequence, image-based systems are even of high relevance when larger areas are averaged, since

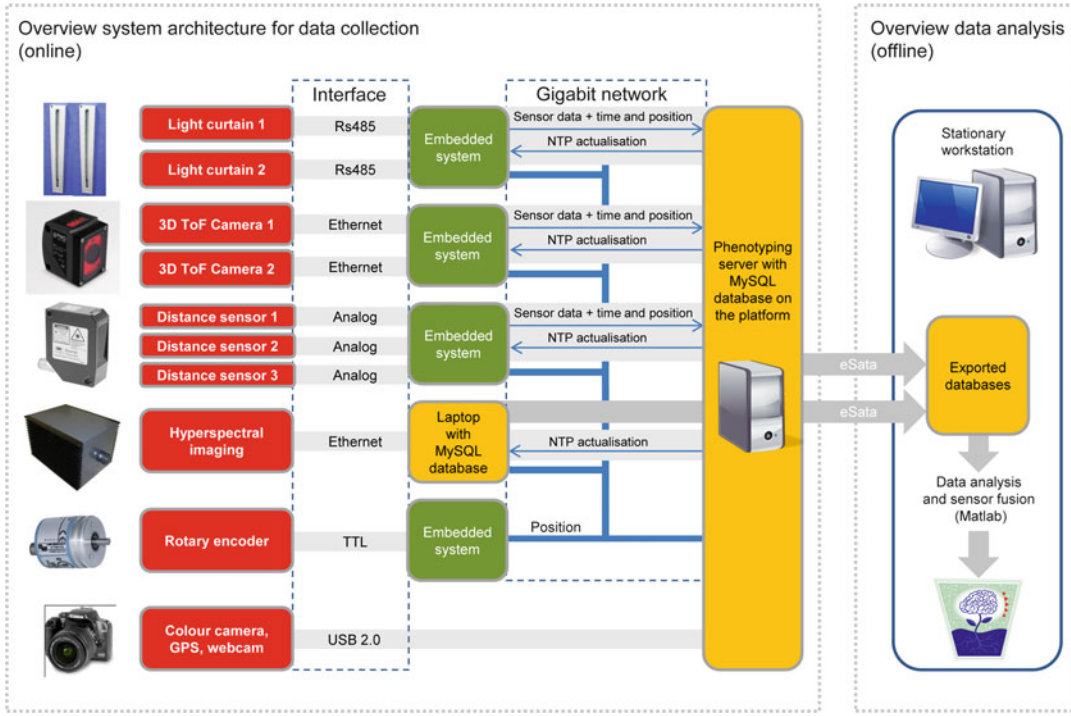

Fig. 4.1 System architecture for data collection and analysis of a multi-sensor phenotyping platform (Busemeyer et al. 2013a)

they allow a sophisticated filtering of raw data in order to obtain robust and high-quality crop information.

- If the interpretation is related to a very small local area (a square meter or less) or even an individual plant, the corresponding data have to be stored extremely precisely in the online data acquisition process in order to match the data of one or more sensors to the same field position in the offline data analysis. If the technical accuracy is higher as compared to the distance between the individual plants, there is even an option for "individual plant-related" phenotyping. An example is the maize where a combination of sensors and high-resolution GSP sensors has been used for individual plant phenotyping (Fender et al. 2006). In plot-related method, an image-based spectrometer included in a position-sensitive data structure allows the detection of crop and soil and thus a phenotyping interpretation. Moreover, selective options as necessary for disease detection or exclusion of plot edge sections are open.

To take into account the variations in frame size and frame rate, spatial and temporal information have to be stored related to each sensor. The position could be supplied by a GPS system, an encoder, or landmarks, while the time could be supplied by an internal clock signal supplied within the system. The position and timing information consequently allow the matching of sensor data to a field position, thereby taking into account the different mounting positions of the sensors. Figure 4.1 shows an example of a system architecture for multisensory phenotyping (Klose et al. 2010; Busemeyer et al. 2013a). On the left side, different sensors are shown, typically with different electronic data interfaces, such as "Ethernet" or "Analog." In the example computers or embedded systems are used for converting all data to a Gigabit Ethernet network.

All raw data with corresponding time stamps and positioning data are stored online in a database serving as the input data for the offline data analysis.

The goal of the complete data interpretation for phenotyping is the conversion of raw data to crop parameters for trait determination. Thus, digital phenotyping is a strongly interdisciplinary task with respect to the application-oriented knowledge of crop plants (such as from plant breeders or biologists) on one side and engineering- or mathematical-oriented methods on the other side with topics ranging from mechatronics, electronics, communications to algorithms, or artificial intelligence.

In particular, this interdisciplinary aspect is of highest relevance for the conversion of sensor raw data to crop parameters. For example, while taking the crop height, it is obvious that there will never be a universal "crop height sensor." The large variety of crop heights, morphological plant structures, growth stages, or field conditions will lead to specific solutions based on sensor technologies shown in Sect. 4.2. The selectivity of each sensor signal varies with respect to a given plant characteristics; moreover, each sensor is effected by specific influences from the environment. As a consequence, an individual sensor might lead to misinterpretations, whereas the combination of different sensors typically results in a higher selectivity. This concept of a multi-sensor system for crop detection has already been proposed in the late 1990s (Ruckelshausen et al. 1999) and has been continuously applied for field phenotyping (see, e.g., Montes et al. 2007; Comar et al. 2012; Busemeyer et al. 2013a). For breeding for novel traits, the concept of digital phenotyping based on sensor fusion including new technologies is a key component to measure the large number of plants and its parameters (Fiorani and Schurr 2013).

The initial idea is the correlation of different parameters extracted from individual sensor raw data with individual crop properties, whereas a sensor can support more than one parameter. Looking at an image of a color camera, for example, several parameters such as the number of pixels in a given color space range (correlated with the crop surface coverage) up to an object identification of a leaf or an ear can be used. As essential for phenotyping in general, sensor-specific field calibrations have to be carried out. In a "sensor-crop matrix" (Dzinaj et al. 1998), all parameters (both from sensors and crops) can be correlated, and promising options in combining sensor data can be established on a manual base. If test measurements are available, mathematical algorithms suitable for sensor and data fusion (Mitchell 2007) can be applied to select the most robust combination of sensors for trait determination or exclude sensors with low selectivity in order to reduce complexity or costs. Already existing data or information (a priori information) is combined with sensor data and can thereby increase the robustness of the algorithms.

The extraction of parameters – relevant for phenotyping – out of digital image-based information is strongly supported by a huge number of algorithms (Davies 2012), which can be applied by commercial or open-source image processing libraries. In a low-level data analysis of images, statistical data can be extracted and used for crop property correlation. An example is the measurement of the area (or percentage) of a field, which is covered by plant material or earth. The image processing is very fast in this case and can be used for online actuator control; however, for phenotyping, the information is typically limited and does not exploit the potential of the data available. Using image processing algorithms for image segmentation and object detection offers a high potential for detecting crop properties. The image processing for indoor phenotyping with individual crop plants is complex however, as compared to outdoor phenotyping which is comparably straightforward. In a laboratory or greenhouse, the individual plants can be separated from each other like in an industrial production environment, and triggered image capturing with ideal light conditions can be performed. Going to field phenotyping, there are many disturbing influences. The major

problem for individual plant detection is to know where the plant is located; in particular the situation of overlapping plants strongly limits applications. The disturbances related to the measurement process can be addressed in terms of the two following categories (Busemeyer 2013).

4.3.1 Factors Having a Direct Influence on the Sensor Raw Data Generation

Environmental impacts, for example, sunlight, dust, or moisture, can strongly influence the sensor signal or even prevent any reasonable data analysis. The selectivity for the different influences strongly depends on the sensor measurement principle, the housing, and the system integration in the phenotyping platform. By adapting the mechanical construction (e.g., by reducing the influence of direct sunlight) or the inclusion of additional redundant sensors the robustness can be increased. For the phenotyping process an online detection of the data quality is recommended in order to avoid measurements without collecting high-quality data. Traffic light information systems for nonfunctioning (red), problematic (yellow), or functioning (green) can be used for the human-machine interface.

4.3.2 Factors Which Temporally Change the Condition of the Plants

This category contains factors like strong wind, heavy rain, or mechanical influences of the phenotyping platform itself. The latter ones should be avoided by a corresponding design of the platform matching to the crop structures. The ground clearance should be sufficient, and variations of the soil level should not cause strong vibrations of the platform or misalignments. The automatic detection of plot damages could be helpful. Information might be included via a human-machine interface.

4.4 Digital Phenotyping in Crop Fields

Image-based sensors and data fusion concepts are applied widely under laboratory or greenhouse conditions. The growth conditions for crops and its disturbances in the field (mentioned in the previous section) are strong needs for outdoor experiments and robust digital technologies. Aspects related to control versus natural environments are discussed in detail in Chap. 3. However, this section has focused on digital field phenotyping for the development of the digital imaging technologies and system architecture laboratory test setups. Examples are robotic arms, carousels, or test vehicles. Figure 4.2 shows a conveyor belt, where crop

Fig. 4.2 Laboratory setup for multi-sensor phenotyping development. A conveyor belt with defined (variable) speed represents the field, while fixed sensors are evaluated with respect to their raw data and sensor fusion options in the follow-up data analysis (Photo: University of Applied Sciences, Osnabrück 2013)

plants or test structures are moved with defined (variable) speed which offers the opportunity to evaluate the usability of new or existing sensor technologies for different application scenarios. Moreover, the quality of sensor and data fusion algorithms can be evaluated under ideal conditions as well as the accuracy of the measurements by repeating measurements.

In digital field phenotyping, we focus on the sensor-based raw data acquisition for the usage in field platforms up to the data analysis resulting in crop properties, ranging from crop height up to complex parameters like biomass (Thomas 2006). The interpretation of the digital phenotyping results to assess the dynamic genetics of complex traits and setting up field trials with respect to a statistical point of view have been discussed elsewhere in various chapters of this book.

It is possible to install sensors permanently in the field or at fixed outdoor frames (like a rain-out shelter). However, in this chapter, we focus on dynamic platforms serving as sensor carriers for nondestructive dynamic field measurements. While existing technologies in agriculture such as satellite remote sensing (see Chap. 13) or tractors can in principle be used as phenotyping platforms, they typically do not fulfill quality requirements for plant breeding processes. Therefore, phenotyping-specific platforms have been designed in the last years and applied to various crops (see Chap. 18). These platforms are required to fulfill the following conditions:

- The mechanical concept includes the sensor systems, data management, power supply, communication technologies, and human-machine interface.
- The sensor positions are flexible and/or adapted to crop properties.
- The platform is robust with respect to field conditions and environmental impacts.
- The speed of the platform compromises high-throughput demands with big data acquisition rates.
- Failure of technological components, such as sensors, is automatically detected and communicated to the user in order to avoid mistrials.
- The platform does not damage crop plants.

Next to these phenotyping-related aspects, the platform has to fulfill legal requirements and safety aspects. The latter ones could be complied by the integration of a human being (at the platform or a remote control place) or by using certified automation systems.

The recently developed phenotyping platforms are mainly ground-based systems, driving through the outdoor field or experimental plots and scanning small crop regions or individual plants (Montes et al. 2011; Wunder et al. 2012). Typical concepts for state-of-the-art developments of dynamic field-based phenotyping platforms are shown in Fig. 4.3.

- A phenotyping carrier is attached to a standard tractor for field trials (Mistele and Schmidhalter 2010; Busemeyer et al. 2013a).
- A specific human driver-based vehicle is designed for phenotyping (Kipp et al. 2014; Comar et al. 2012).
- The systems are integrated in an autonomous field robot platform (Griepentrog et al. 2010; Ruckelshausen et al. 2009).

The phenotyping platforms collect and store raw data for experimental plots, small regions, or even individual crop plants. As described in Sect. 4.3, the spatial and temporal resolutions of the raw data have a strong impact on the data management concept. Thus, the selection of sensors and the statistical models for crop parameter extraction strongly depend on the planned interpretation in terms of a complete field, a site-specific view, a plot-related interpretation, or an individual plant treatment. If high-resolution imaging tools are used, the high-volume information can be filtered and averaged for a larger region, thereby typically resulting in reduced data of high quality. If the raw data are already integrated and thus averaged, for example, for a nonimaging spectrometer, the filtering options are strongly limited and thus the quality is typically lower. Another important aspect in digital field phenotyping is related to the sampling process. Is it necessary to measure all positions along the platform path, or would it be sufficient to collect samples along the way? The latter

Fig. 4.3 Realized concepts of digital field-based phenotyping platforms. On the *left side*, the multi-sensor phenotyping BreedVision carrier is attached to a tractor (Photo: University of Applied Sciences Osnabrück, Germany 2012), the human-based special purpose vehicle PhenoTrac 4 is shown in the *middle* (Photo: Technical University Munich, Germany), and the autonomous field robot for phenotyping applications BoniRob is shown on the *right side* (BoniRob; Photo University of Applied Sciences Osnabrück, Germany)

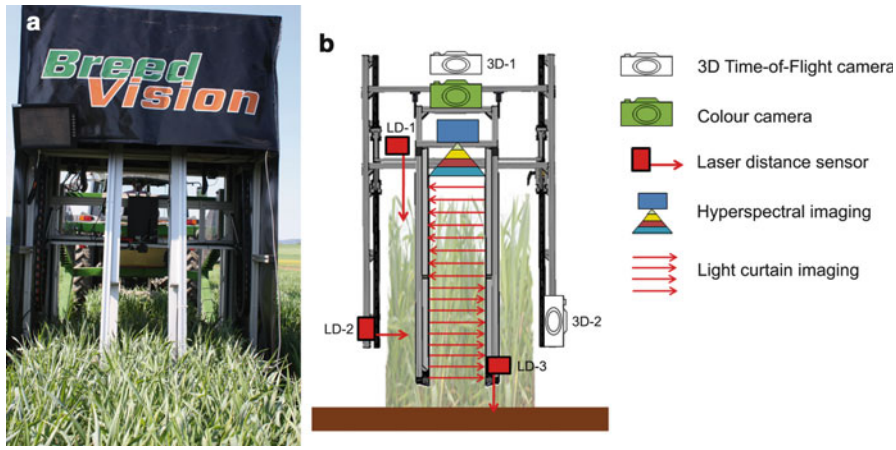

Fig. 4.4 Tractor-mounted phenotyping platform BreedVision from rear view (**a**) and the mounting layout of multi-sensor attachments (**b**). The light curtains are designed to pick up in case of soil contact (Busemeyer et al. 2013a)

sampling version is by far easier to realize, while for a complete sensor tracking, the frame rates for each sensor have to be adapted to the speed of the phenotyping platform or vice versa. High spatial and temporal resolutions and high speed are conflicting targets.

The integration of sensors into the mechanical platform has to take into account the variability of crop and field conditions. For detecting morphological structures, the size range of a crop, for example, dominates the selection and positioning of a sensor. For optical image-based systems like camera variations of the distances from the sensor to the object are relevant with respect to focusing the optics to get interpretable raw data. Figure 4.4 shows the field phenotyping platform for triticale ranging from heights of a few centimeters up to about 2 m (Busemeyer et al. 2013a). The sensor fusion concepts include light curtains, placed left and right of the selected measurement area, while most of the other sensors are positioned for a top view. Sensors like spectral imaging devices or 3D time-of-flight cameras are based on active (in some cases synchronized) illuminations, thus cross sensitivities have to be taken into account in the phenotyping platform design phase. As can be seen in Fig. 4.4, the sensor region is partly shaded to reduce the impact of sunlight variations. Moreover, it is important to take care about

humidity or even rain as well as long-term effects of dust. In particular the effects of these environmental impacts could be crucial for optical sensors. As typical for all sensor applications, there should be a clear information to the user in case of a failure; however, it would be very helpful to receive a warning prior to loosing data. Using the time available between experimental plots or in a headland area, algorithms can be performed to generate warnings for the user. For example, in the case of a light curtain, dust-covered light barriers can be detected and the user can react. Since all data are available in a digital format, they can be used during the data analysis for interpreting possible inconsistencies.

The phenotyping platform for generating field-based raw data is the key component for the digital phenotyping. The platform typically includes several sensors, in particular image-based devices. As compared to a human in the role of a "classical phenotyping platform," the data are generated continuously with the same quality if the abovementioned measures for robust data acquisition are taken into account. There should be no variations as in the case of comparing one human expertise with the other; moreover, human experts might get tired and quality might change during data capturing. Nevertheless, the accuracy of a technical system has to be evaluated in order to avoid overinterpretation of measurement results in terms of crop parameters while it is only a noise signal due to variations of data acquisition and algorithms. As a consequence, a new procedure, called "technical repeatability" is proposed by Busemeyer et al. (2013b). As compared to the agronomic repeatability, where, for example, experimental plots with the same genetic crop variation are repeated within a field, the technical repeatability includes a double measurement for a selected number of experimental plots (or field regions). The variations of these measurements represent the precision of the entire phenotyping procedure. Figure 4.5 (left side) shows an example of a series of measurements during three harvesting periods. The height of triticale is measured with a technical repeatability resulting in an R^2 of 0.99. This number does not replace the calibration for the plant height with an independent method but serves as a selective tool for interpreting differences in crop traits. An example for comparing human phenotyping (using a mechanical scale) with digital phenotyping (using light curtain data) is shown in Fig. 4.5 (right side). The correlation is very good ($R^2 = 0.96$), however – due to the human repeatability – somewhat lower as compared to the technical repeatability.

Except the aspect of the technical repeatability and the availability of high-resolution digital data, the agronomic principles of the phenotyping procedures are still similar. This includes trait calibration procedures which have to be carried out trait-specific as well as sensor-specific. The calibrations are applied to the trait determination procedures. As usual, the effects of the environment to the habit of the plants have to be taken into account by accumulating data for validation and calibration at different locations. The experimental design for precision in phenotyping and further information about screening plant canopy parameters are described in Chaps.17 and 6, respectively.

Figure 4.6 (left side) shows the results for the correlation of observed and predicted dry biomass yield for three harvests (Busemeyer et al. 2013b). The measurement data for the digital phenotyping are based on light curtains and laser sensors. The potential of sensor and data fusion is demonstrated by the inclusion of spectral imaging as an additional image-based sensor (Fig. 4.6, right). By using a moisture index (a ratio of selective wavelength), the relative moisture content can be determined point wise and a more appropriate number of the dry biomass can be determined. The resulting correlation with a $R^2 = 0.92$ shows a significant improvement as compared to $R^2 = 0.77$ without this sensor fusion component.

4.5 Future Trends in Digital Phenotyping

The introduction of digital phenotyping to the practice is still on the go. In research and development, there is a tremendous increase of

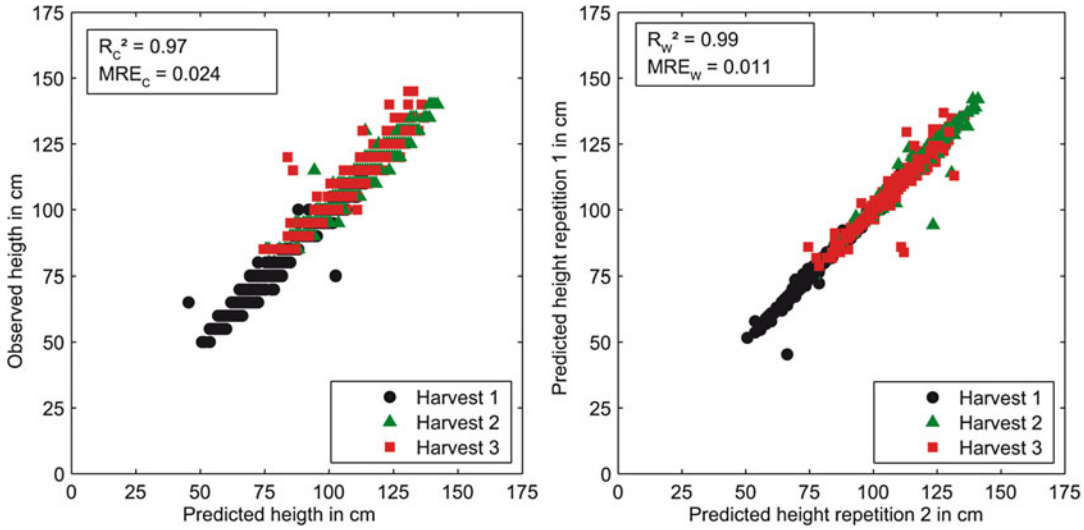

Fig. 4.5 The technical repeatability of a digital crop height measurement with image-based light curtains for a series of field tests (*Triticale*) is shown on the *right side*. Two measurements of the same plot show a correlation of $R^2 = 0.99$, which represents the technical accuracy of the digital phenotyping platform. On the *left side*, the digitally measured crop height is correlated with a human one, resulting in an R^2 of 0.97, thereby reflecting the lower human repeatability (Busemeyer et al. 2013a)

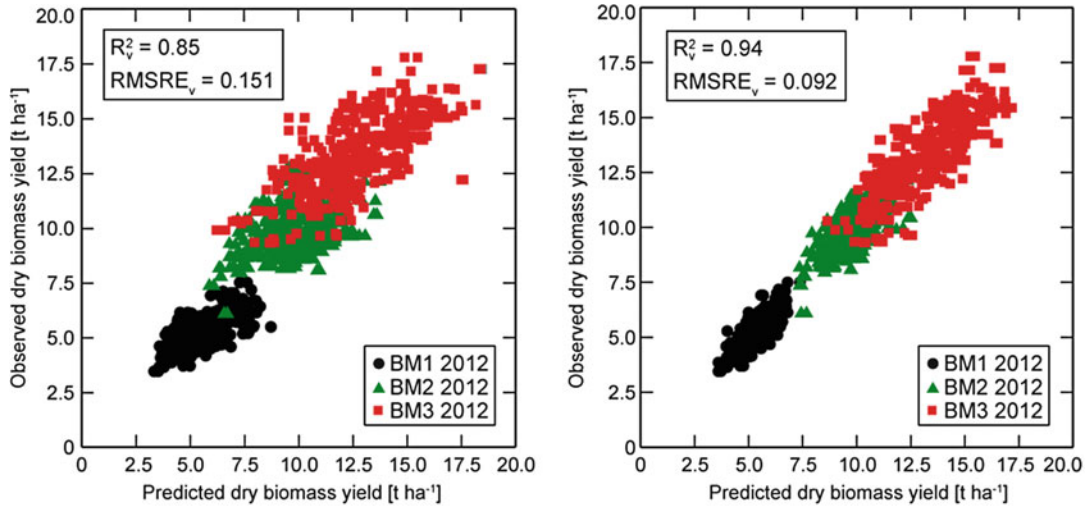

Fig. 4.6 For a set of three harvests, the biomass predicted by digital phenotyping is correlated with the observed values obtained by harvesting, drying, and weighing (*left side*). The data processing is based on light curtain and laser distance measurements and thus ignores the moisture portion. On the *right side*, the image-based data of a spectral imaging system have been taken into account to correct for the moisture content. The sensor and data fusion significantly increases the correlation (Busemeyer et al. 2013b)

activities and projects on this topic as indicated by various networks, workshops, and conferences (see, e.g., IPPN 2013 and links therein). However, having a look at products or services, only few options are available. Moreover, most of them still have a major focus for

indoor or greenhouse phenotyping, while field phenotyping is still in the beginning (see, e.g., LemnaTec 2013; PhenoFab 2013). On a first view, this appears unexpected, since the pros of digital field phenotyping are generally accepted: the uniformity and objectivity of the measurements, the availability of digital data for further data analysis and documentation, a high automation level thereby reducing labor and time consumption, and the availability of spatial and temporal high-resolution selective data, in particular of image-based devices. However, the complexity of the technologies for data acquisition as well as analysis is very high and has thus limited the spread of digital phenotyping (Eberius and Lima-Guerra 2009). The costs of the technology modules are high, and there is still a lack of robust (good) algorithms for outdoor measurements under interfering variable influences. Moreover, the knowledge about plant growth strongly limits the interpretation of phenotypic data. Innovative and robust image-based technologies in field measurements can support the finding of correlations between various influencing factors and thereby the development of analytical models for plant growing (Poorter et al. 2010; Eberius and Lima-Guerra 2009). At the moment, the complex answer to the question "How does a plant grow?" is typically answered by plant breeders, biologists, and farmers with "It depends on. . .." Due to the increase in the understanding of the processes in nature on one side and the improved digital phenotyping technologies on the other side, there is the potential to give more analytical answers in the future.

4.5.1 Sensor and Data Fusion

The sensor and data fusion (see Sect. 4.3) is the most promising approach in digital phenotyping since varying selectivities of an individual sensor can be compensated to a large extent. The algorithms, however, become complex and rely on a large number of crop- and sensor-specific calibration data from field measurements. Thus it is of high relevance to separate data acquisition of raw data from the transformation algorithms for crop trait determination. The quality and resolution of the raw data will always be a topic for improvements in digital phenotyping. Recent developments for new sensor concepts will find their way to field phenotyping applications. Examples are the terahertz technology, magnetic resonance, radar technology, and multi-reflection ultrasonic sensors or multifocus cameras (for supplying focused images for different camera-crop distances for a single image capturing). Moreover, multiview cameras, in particular 3D devices, offer the opportunity to measure details of individual plants. The subsequent algorithms have to take into account the unlimited variability of crop and field situations. Solutions for robust algorithms are applied for artificial intelligence methods; the attachment of quality attributes to algorithms or semiautomatic data analysis including a human:

- Algorithms: For instance, probabilistic algorithms – for example, used in probabilistic robotics (Thrun et al. 2005) – are suitable for the problem.
- Attributes: For image analyses, quality parameters can be defined and transferred into a brief traffic light information, where "green" represents a high quality. The attributes can weigh the influence of an individual algorithm within the sensor fusion concept.
- Semiautomatic analysis: The database containing the raw data can be semiautomatically used for analyses. For example, a user can select individual crops or field sections for the subsequent automatic analysis.

Typically, the raw data are analyzed and calibrated in terms of crop traits in a direct way as shown in the example of determining the crop height in Fig. 4.5. However, for more complex parameters, indirect options can support parameter extraction. Examples could be screening methods for phenotyping diseases (see Chap. 9). The local detection of very small spots (in the range of square millimeters) in the field as indicators for diseases is a high challenge, in principle spectral imaging technologies are

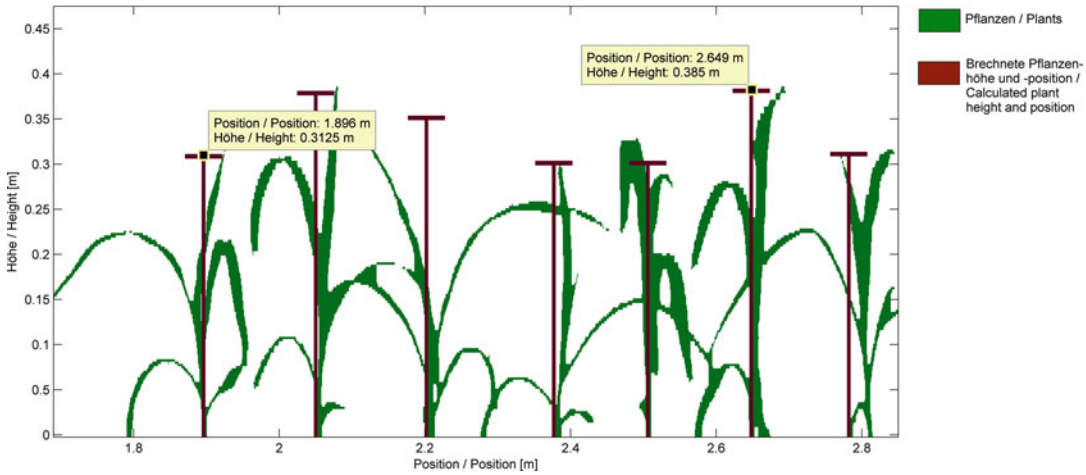

Fig. 4.7 Measured raw data (*green*) of a light curtain in maize (Wunder et al. 2012). Based on sensor fusion with an RTK-DGPS, it is possible to redetect the individual crop plants and model crop properties such as the height (*red*). The technology demonstrates the potential of individual plan phenotyping with an autonomous field robot

suitable for such a procedure, but the small size and the corresponding field of views are disadvantageous. If indirect parameters – such as the plant height, the number of plants, or the relative biomass – are easier to access, the areas suspiciously infected can be marked in a digital map, and a human expert can evaluate the field situation. By iterative human-machine interaction, the quality and degree of automation of these processes can be increased.

4.5.2 Individual Plant Phenotyping

The need for robust digital phenotyping does not only affect the sensors and algorithms but also the mechanical platform and in particular the data management. The need for high-throughput platforms with reasonable costs and high quality is a major requirement for digital phenotyping. While ground-based platforms are used in presence, the availability of unmanned autonomous aerial platforms (UAV), in particular low-speed and height quadro- or octocopter devices, has become of great interest (Zude 2013 and references therein) and offers future options in phenotyping. Due to the limited top view and additional disturbances caused by the flying platform, ground-based systems still are in favor. For high-throughput plant phenotyping, autonomous or remote-controlled field robots (see Fig. 4.3, right) can be of high relevance in the future (see also Chap. 7). With sensor and data fusion including high-resolution GPS information, even individual plant phenotyping is an option. In this case, crop growth behavior can be evaluated for selected single plants or regions (Wunder et al. 2012). Figure 4.7 shows raw data of light curtain measurement in maize. The data show the side view of the crop plants like a shadow. Together with an RTK-DGPS, it was possible to redetect the individual plant several times. The positions as well as the modeled height of the individual plants are marked in Fig. 4.7. For further data analysis and visualization, existing GIS tools – meant for site-specific applications in precision farming – can be used; in this case, the "site" corresponds to an individual plant. Measurement data, images, or statistical data of field regions can be viewed and used for further processing and evaluation (see Fig. 4.8).

4.5.3 Virtual Phenotyping

As in many other disciplines, simulation can play an important role with respect to understanding

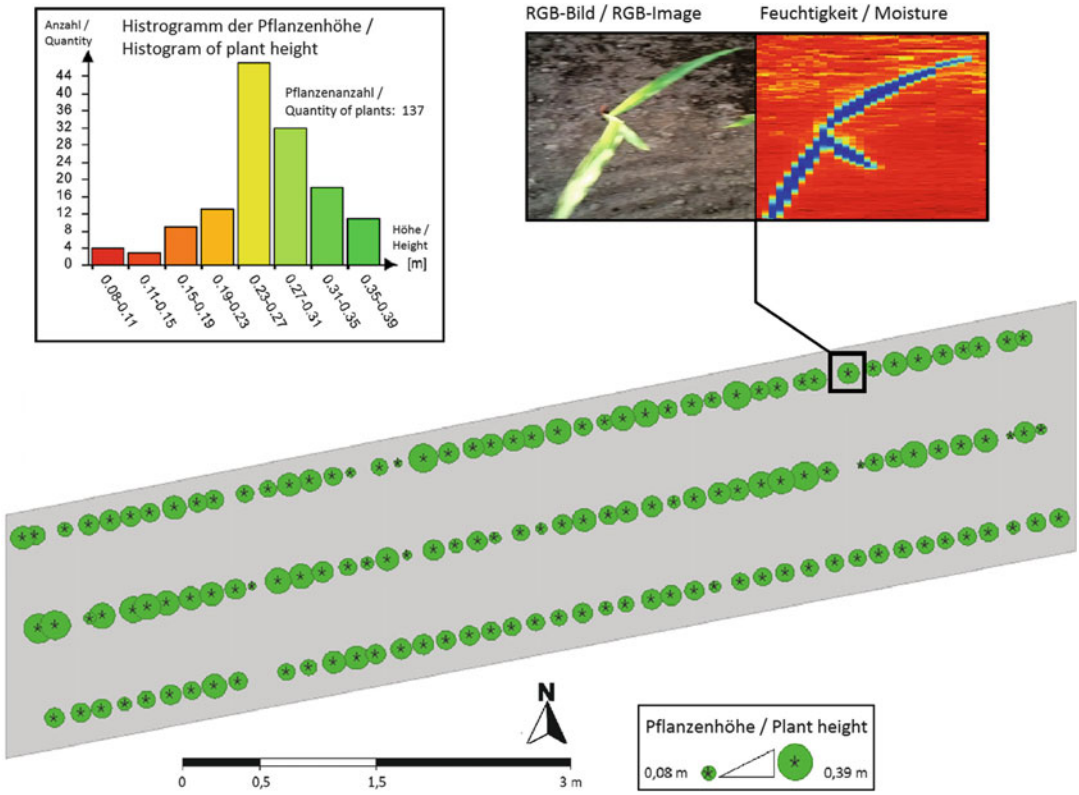

Fig. 4.8 GIS map of three maize rows as measured with the autonomous phenotyping platform BoniRob. The data and images of individual crop plants can be visualized; moreover, statistical analysis of regions can be performed (Wunder et al. 2012)

processes or develop new ones. This aspect has not yet been focused intensively; however, most likely, it will be a topic of high relevance in the future. Due to the infinite options of sensor selection, sensor positioning, crop states, and environmental conditions, it could be very helpful to evaluate the quality of image-based sensors and sensor fusion concepts prior to the field trial. For example, the options of integrated simulation environments – such as ROS (Robotics Operating System, ROS 2013) – have already been applied for simulating the data acquisition for different sensors in a field-like situation (Tsukor et al. 2012). For such a virtual phenotyping, the sensor characteristics have been implemented, and the simulated measurement data are stored in a database which is identical to the real one for the field measurements. Figure 4.9 shows the schematic process of virtual phenotyping, where sensors are implemented in a carrier, such as the autonomous field robot BoniRob or a tractor-mounted device. Due to the infinite possibilities of sensor characteristics or positioning, the simulation has the potential for optimizing sensor acquisition. Moreover, the robustness of algorithms for crop traits can be evaluated prior to field measurements. Since sensor and data fusion has a high complexity, virtual phenotyping can be applied to remove sensors with low selectivity and thus reduce complexity as well as costs for sensors and system technologies. On the other hand, the benefit of new sensors can be evaluated.

The idea of virtual phenotyping has already come up in clinical therapy (Perez-Elias et al. 2003), where the genetic information is transformed into the most likely associated phenotype. In crop science, the expression virtual

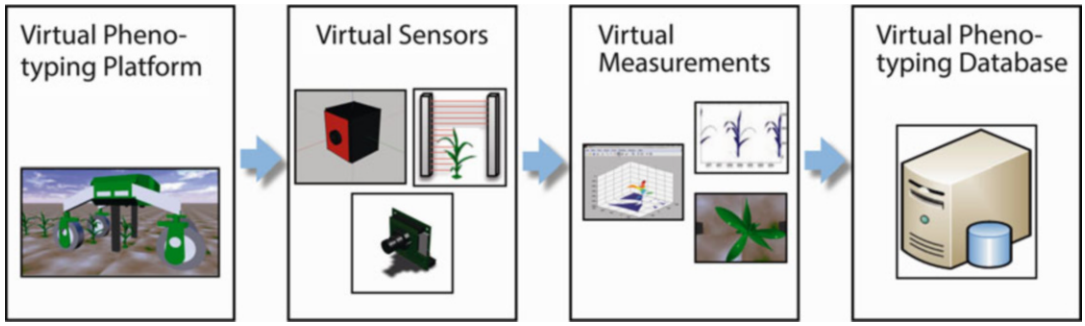

Fig. 4.9 The concept of virtual phenotyping is shown (Tsukor et al. 2012): the phenotyping platform attached with several sensors is simulated within the ROS framework (Robotics Operating Systems, ROS 2013). The virtual crop plants can be "measured" with the simulated sensors, and the data can be stored in the same database as the real measurement data

phenotyping has been used first referred to real measurements based on near-infrared reflectance spectroscopy (NIRS) and Fourier transform infrared spectroscopy, while the virtual character is interpreted in contrast to the biochemical analysis (Donnison et al. 2009). Having a look at state-of-the-art digital phenotyping, however, the interpretation of virtual phenotyping in terms of a complete simulation of data acquisition appears appropriate.

4.5.4 Multiuser Digital Phenotyping

The future challenges with respect to sensors, platforms, algorithms, and simulation have been addressed above. In particular for digital phenotyping, the application of several (image-based) sensors results in a huge amount of raw data in the range of gigabyte per plot. These raw data from different locations, diverse platforms and users, different time points, and various crops have a high value, which can be potentiated if the data are available for multiuser applications. Moreover, using earlier date data for applying new algorithms or generating a broader base for interpretation is of high interest. As a consequence, data management is of highest interest in digital phenotyping. In the first step, the conversion of different data formats or steps toward more standardized data is in focus. If the data are available in a database various algorithms and target directions can be applied, for example, by looking at the measurements in terms of a data warehouse concept (Billiau et al. 2012).

4.6 Conclusion

In nutshell, the phenotyping and genotyping databases have to be linked together (Cobb et al. 2013; Perez-Elias et al. 2003). This tremendous task can only be solved by strongly interdisciplinary approaches with experts from different disciplines. The technological tools for getting reliable field data or the data management and interpretation concepts have similar complexity as the genotypic interpretation. Taking into account the complexity of crops and nature, digital phenotyping – including image-based sensors mounted on platforms – robust multi-sensor and data fusion, and simulated-based methods are key components for today's and future field phenotyping.

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Imaging Methods for Phenotyping of Plant Traits

5

David Rousseau, Hannah Dee, and Tony Pridmore

Abstract

This chapter introduces the domain of image analysis, both in general and as applied to the problem of plant phenotyping. Images can be thought of as a measurement tool, and the automated processing of images allows for greater throughput, reliability and repeatability, at all scales of measurement (from microscopic to field level). This domain should be of increasing interest to plant scientists, as the cost of image-based sensors is dropping, and photographing plants on a daily or even minute-by-minute basis is now cost-effective. With such systems there is a possibility of tens of thousands of photographs being recorded, and so the job of analysing these images must now fall to computational methods. In this chapter, we provide an overview of recent work in image analysis for plant science and highlight some of the key techniques from computer vision that have been applied to date to the problem of phenotyping plants. We conclude with a description of the four main challenges for image analysis and plant science: growth, occlusion, evaluation and low-cost sensor vision.

5.1 Introduction

In plant phenotyping, large cohorts of plants are measured, to determine characteristics of the way in which the plants' phenotypes are expressed. Imaging techniques could become tools of choice in this context, since they allow non-destructive and non-invasive measurements capable of reproducing the eye of experts and can even overcome human eye performances when gazing outside of the visible spectrum, for example, into the infrared part of the spectrum or investigating microscopic scales. Measurements can be made for different purposes (genetics, plant breeding,

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aesthetic matters when considering ornamental plants), in different environments (controlled and uncontrolled, single plants or plots) and with a wide variety of sensors (X-ray tomography, fluorescence imaging, visible light, infrared, magnetic resonance imaging, terahertz imaging). In some systems, sensors are integrated into the surrounding infrastructure (moving field, moving camera, drones); in others the sensors may be portable (e.g. handy cameras and smartphone-embedded sensors). All these systems generate digital images which contain information on plant structure, function and growth. Whilst there is a real sense in which an image is a measurement, the more interesting task lies in extracting quantitative measurements from images which have intrinsic value for biologists. When such a procedure becomes fully automatic, the throughput of plant phenotyping systems will leap up, enabling large-scale monitoring of plant growth, escaping the current phenotyping bottleneck involving hand measurement of individual plants.

This chapter focuses on the automatic recovery of quantitative data on plant structure, function and growth from image data. This is a large area and we restrict discussion to images acquired in controlled environments under illumination in the visible spectrum. Though more than one plant may be visible in any given image, we assume that the plants are separable, i.e. we consider plant, not canopy, phenotyping. Several reviews of related areas from a biological point of view have been published in recent years (Furbank and Tester 2011; Fiorani et al. 2012).

It is important to emphasise from the outset that we believe computer vision for plant science should be a two-way relationship benefitting both parties. There are plant science questions which can only be answered with large-scale automatic measurement of phenotypes, and there are vision questions posed by such systems that will require development within the field of computer science too. We therefore propose a review of the top-level questions facing the designer of an image-based phenotyping system, before introducing some of the specific image analysis challenges to be overcome and then concluding with some open questions in computer vision for plant science. In this way we hope to introduce the concepts and questions from image analysis that will enable plant scientists to work well with vision scientists, bringing progress to both fields.

5.2 Image Capture: Designing a Phenotyping System

Image-based phenotyping methods share common structure and components. This includes the fundamental acquisition components of a computer vision system: lighting, optics and imaging technology. In this section we cover some basics concerning the choice of these components and discuss their interrelations with respect to the targeted phenotyping traits.

The current technologies available for lighting in computer vision are given in Table 5.1. Halogen and fluorescent lights are mainly used in growth chambers. These techniques are modulated at the frequency of the electrical network (50–60 Hz) and cannot be used in pulsed mode. As a consequence, fluorescence imaging techniques well known in plant science (see Papageorgeou and Govindjee 2004 for a review) can only be developed with LED technologies.

The choice of the wavelength of lighting is important since it affects the contrast observed in

Table 5.1 Overview of lighting technologies used in plant science

Technology	Advantages	Drawbacks
Halogen	High intensity	Short life cycle, high volume, high cost, works in continuous mode
Fluorescent	Very diffused light	Short life cycle, high volume, high cost, works in continuous mode
LED	Low volume, low energy consumption, long life cycle, continuous and pulse mode	High cost on large surface

the acquired image. For illustration, let us imagine a scene containing green and red leaves, where the informational task would be to separate, automatically through the use of computer vision, the green leaves from the red leaves. Choosing a white light would send the contrast between the two populations into the colour domain requiring a trichromatic RGB sensor, whilst choosing a green light could enable the discrimination of the green leaves (appearing bright) from the red leaves (appearing dark) with a simple monochromatic grey-level sensor.

Halogen and fluorescent lights have large wavelength bandwidths covering the visible spectrum, whereas LED lighting can either have a large bandwidth or it can instead be constructed with a narrow bandwidth restricted to ranges as narrow as 10–30 nm. It is therefore easy to select adapted wavelength bands with LED technologies. However, there are also bandpass, short-pass or long-pass filters that can be mounted on large bandwidth lighting systems (see, e.g. http://www.edmund.com) which can perform similar restrictions on the light that reflects from the biological sample or plant. Wavelength choices adapted to plant imaging have been reported for various domains of plant science including the monitoring of plant growth or of abiotic and biotic stresses (Sankaran et al. 2010; Fiorani et al. 2012).

The design of optimal light sources for given applications of plant imaging is an open question, but reference to previous applications in this domain may guide choice. For unreported applications of plant imaging, it is possible to determine the optimal wavelength of lighting by using hyperspectral imaging. This imaging technique acquires, like a spectrometer but in 2D, multiple (typically some hundreds of) wavelengths covering the entire visible spectrum and possibly the near-infrared spectrum with a spectral resolution of a few nanometers, i.e. much larger than any light source shown in Table 5.1. Hyperspectral imaging hence produces images of the spectral reflectance of the observed scene. The hyperspectral reflectance of leaves can be also simulated with numerical models. This has recently been made

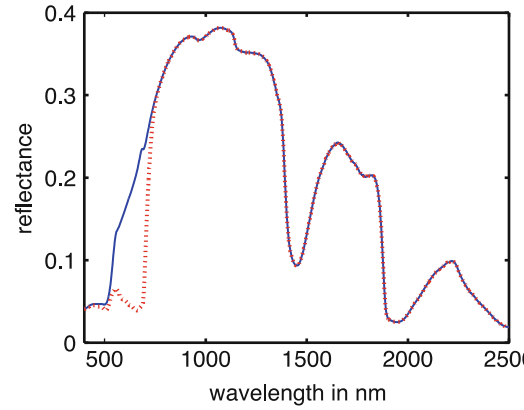

Fig. 5.1 Reflectance spectrum of a control leaf in *dash-dotted line* and of a leaf with a loss of chlorophyll in *solid line*

available online with the website http://opticleaf.ipgp.fr/. From analysis of the reflectance spectrum in those areas to be separated within the visual scene, it is possible to determine the best wavelengths in the sense of the theory of detection (Kay 1998). It is then only necessary to light the scene with these best wavelengths to constitute optimal lighting systems. For illustration, we give the reflectance of a control leaf and a leaf suffering from a loss of chlorophyll in Fig. 5.1. In this case, the optimal wavelengths for discriminating between the two spectra are located in the range 500–700 nm, and lighting these samples with wavelengths within this range will greatly simplify later vision tasks.

The geometry of light is another important parameter that can be optimised. This geometry corresponds to the dimension and position of the lighting system(s), in relation to the observed scene and camera. It influences the homogeneity of contrast expected from the choice of the wavelength in the light source. A bad choice of light geometry causes shadows in the acquired image; dealing with shadows in computer vision is a research topic in and of itself (Sanin et al. 2012; Dee and Santos 2011), and if this can be avoided at the capture stage, it greatly simplifies further processing steps.

Various lighting geometries are used in computer vision as given in Table 5.2. Some of these techniques are already used in plant imaging. For

Table 5.2 Main geometries for lighting systems and corresponding applications

Geometry	Typical applications
Annular: ring fixed on the camera	For high intensities
Backlight: object placed between light and camera	For shape recognition from transparency
Diffuse: reflectors placed around the observed scene	To produce uniform diffuse light
Scattering: lights scatter on the edges of the scene	To enhance edges

instance, backlights are used to monitor seedling growth (French et al. 2009; Subramanian et al. 2013; Benoit et al. 2013) and diffused light is used in the imaging cabinet (http://www.lemnatec.com/). The techniques given in Table 5.2 are of interest when it is possible to position the lighting system in relation to the observed plants. This might not be the case when imaging has to be done in the growth chamber or even in the field. In these cases, time-gated lighting can be a good solution to get rid of the ambient light. This involves sending a brief and intense flashlight onto the scene in synchronisation with the shutter of the camera. The sensor of the camera is relatively less sensitive to ambient light during the flashlight.

Various parameters influence the choice of optics like the magnification, the depth of view and the minimal distance to the object, which are all accessible from Descartes' law on lenses (see Bass et al. 2009 for a tutorial). In short, the equation of the lens gives

$$\frac{1}{f} = \frac{1}{u} + \frac{1}{v},$$

where f is the focal length of the lens, u is the distance between a point P on the object to the optical centre of the lens and v is the distance between the optical centre of the lens and the focalisation point associated with P. The focal length of a lens is expressed in millimetre. The choice of this parameter is linked to the dimensions of the scene to visualise. Each focal distance corresponds to an angle α

$$\alpha = 2a \tan\left(\frac{d}{2f}\right),$$

with d being the diagonal size of the sensor (in millimetres). A large scene is acquired with a short focal length, whilst a small scene requires a large focal length. Considering this in the specific context of plant imaging, if multiple plants are to be imaged at the same time, the focal distance f governs the throughput of the phenotyping system. For a given size of sensor associated with the lens, the larger the scene imaged, the larger the throughput but also the smaller the number of pixels to be associated with each plant. As discussed by Belin et al. (2011a), there is a trade-off between the throughput and the accuracy of the measurement, and ideally this should be addressed by considering the final phenotypic trait to be extracted.

Another parameter, particularly important when considering adult plants, is the depth of field (DOF). This corresponds to the distance in the object space for which the point P appears focused on the sensor plane. For a lens of diameter D, the DOF depends on the focal length f, the relative aperture of the diaphragm $A = f/D$, the distance u between the object and the lens and Pix the length of a pixel (assumed square here):

$$\mathrm{DOF} = \frac{2A \cdot \mathrm{Pix} \cdot f^2 \cdot u \cdot (u - f)}{f^4 - A^2 \cdot \mathrm{Pix} \cdot f^2 \cdot (u - f)^2}.$$

The final decision in terms of capture is the choice of the sensor. This should take into account its spatial resolution, numerical dynamic (number of bits) and spectral sensitivity with respect to the choice of the light source spectrum. Some companies offer expertise to guide these choices in integrated solutions dedicated to plant phenotyping. It is however worth mentioning a move towards low-cost systems, such as Tsaftaris and Noutsos (2009), Chene et al. (2012), de Vylder et al. (2012) and Wang et al. (2012), which use consumer cameras and open-source software. It is also interesting to note from this perspective the recent initiative

to list open-source software on the web for plant image analysis which can be found at http://www.plant-image-analysis.org/.

The description of the constitution of machine vision system in this section may seem very linear. However, the interrelations between task, environment and method complicate the design of image-based plant phenotyping tools. It is often not immediately obvious, particularly to the non-specialist, how to identify what the assumptions made by an image analysis method are or how well they match the key features of the object of interest and viewing environment. Construction of a plant phenotyping tool is typically an iterative process in which the method and the environment are both tuned until satisfactory and predictable performance is achieved. The tuning process should be informed by an understanding of the assumptions made by the method and ideally driven by sample data from which the degree of fit between object, environment(s) and data can be assessed. One might change the illumination, look at a histogram of grey values to check that it has become closer to the assumed normal distribution and then alter the parameters of the software to deal with, e.g. a general increase in brightness caused by the new lighting configuration. The universal plant phenotyping machine therefore does not exist. To design a plant phenotyping system is to look for a synergy between method, task and environment: the environment needs to be set up in such a way to support the image analysis methods, and the image analysis methods need to be chosen from those which will work in that particular environment.

5.3 Feature Selection: Translating Biology to Image Geometry

Images are sources of information concerning the phenotypic traits to be analysed: if a trait is visible to us, in a captured image, then it should also be possible to extract it algorithmically. Traits could be something as simple as leaf area (which correlates highly with the amount of "green" in a captured, top-down image) or it could be something more technical, like angle between leaves or some measure of the serratedness of a leaf edge. Image processing is thus a matter of information extraction. Various informational tasks can be targeted including object detection, object counting, shape analysis from object segmentation and motion tracking.

For instance, a detection task has to decide if an object is present or not in the image of the scene. This final information can be coded on a single bit: 1 if the object is present and 0 if there is no object. This is very small in comparison with the initial storage necessary to code the acquired images, which often requires megabytes for standard cameras. Image processing can therefore be understood as a reduction of data size whilst preserving salient information, that is, a question of compression. To achieve this, it is necessary to define "the information-carrying feature" to be extracted from the images. These features will come from the expertise of the biologist in charge of the phenotyping. However, biological features (cell, stem, leaf, fruit, branches, etc.) are not trivially understandable in terms of image processing: it is possible to design systems which can extract these features, but they are in no way primitive. An important step, therefore, in the design of plant phenotyping computer vision tools consists of the translation into geometrical terms of the biological features. Since no universal biology-geometry glossary exists, this step has to be approached as a synergy between computer scientists and plant scientists. This section presents an overview of the main geometrical features that can be computed so as to provide plant scientists with the basic vocabulary of image processing.

Objects in image processing are typically described in terms of features. These can be separated into three broad families of features: edges, regions and keypoints. The detection of objects in images is realised by the detection of a set of features characterising the object. For instance, the face detection algorithm (Viola and Jones 2001) now implemented in all basic cameras can be roughly summarised as a system which detects the coexistence of a vertical edge (the nose), a horizontal edge (the two eyes) and then further edges which are found on faces. (Note that

the impressive performance of this face detector is attributable not to the features it uses but to the way in which these features are combined and to the way in which this combination and arrangement of features are learned from observation.)

Edges constitute the boundaries of homogeneous areas corresponding to regions, or they correspond to creases and wrinkles in otherwise homogeneous surfaces, like the ribs of a leaf. Keypoints are points in the images with some predefined local environment; these features are often chosen to maximise the possibility of finding the features again. Hence they can be thought of as representing unusual or visually distinct image areas. Region-based features aim to identify not the boundaries between types of object in the scene but instead to concentrate on finding the (often homogeneous) regions that make up the objects. With respect to usage, when objects are delineated with sharp variations in colour or brightness from their background, edges are a priori more appropriate features to describe the object(s). When the distinction between object and background is more spatially diffused, region-based features may be more likely to constitute good descriptors. Keypoints are particularly useful when occlusions are likely to perturb the detection of the entire contour of an object. In such cases, it is more robust to base the detection of an object on keypoints, as each keypoint only represents part of the object, so it matters less that the object is partially hidden. In practice, it is often the case that a real-world vision problem can be addressed most efficiently with some combination of edges, regions and keypoints.

5.3.1 Edges

Let us consider a grey-level intensity image $I(x, y)$. Edges are extracted by using the gradient in the image. One can define the derivative operation for images along axes x and y:

$$D_x(x, y) = I(x + 1, y) - I(x, y),$$

and

$$D_y(x, y) = I(x, y + 1) - I(x, y).$$

The amplitude of the gradient, illustrated in Fig. 5.2, is then expressed as

$$D(x, y) = \sqrt{D_x(x, y)^2 + D_y(x, y)^2},$$

and the direction of the gradient is given by $\theta(x,y)$

$$\cos\left(\theta(x, y)\right) = \frac{D_x(x, y)}{D(x, y)}, \sin\left(\theta(x, y)\right) = \frac{D_y(x, y)}{D(x, y)}.$$

The derivative operation amplifies small fluctuations which may be caused by noise. To be less sensitive to this noise, it is possible to perform local smoothing before or after the derivative operation.

It is also possible to detect the edges by detecting the zero crossing of the second derivative in x and in y in the images, and

$$L_x(x, y) = 2I(x, y) - I(x - 1, y) - I(x + 1, y),$$

Fig. 5.2 (**a**) An image of leaf; (**b**) the gradient image of leaf; (**c**) a thresholded version of (**b**)

and

$$L_y(x,y) = 2I(x,y) - I(x,y-1) - I(x,y+1).$$

Once the edges are extracted in binary images (such as in Fig. 5.2c), there are several different ways we can choose to represent these contours:

- A Cartesian coding in a table where the coordinate of the pixels in the edges is successively set.
- A Freeman coding of the coding listing the relative angular movement amongst eight possible directions to pass from one pixel in the contour to the next neighbour.
- A parametric representation via a Hough transform or the Fourier coefficients of a Fourier transform of the contour coded as a complex signal $x + jy$. The choice of encoding depends in part upon the informational task to follow. For example, if the aim is finding shapes such as circles (such as when looking for seeds in images), a Hough transform could be useful, but if the aim is to detect or extract repeating features (like the serrated edge of a leaf), a Fourier analysis may be more appropriate. Edges have been extensively used to characterise the shape of leaves and to extract the skeleton of simple plants like seedlings (see, e.g. Du et al. 2007; French et al. 2009; Belin et al. 2011b; Soares and Jacobs 2013; Gwo et al. 2013).

5.3.2 Region

A homogeneous region can be associated either with areas of uniform colour or with a texture. The concept of texture corresponds broadly to basic patterns, deterministically or randomly repeated in space. A great variety of numerical attributes have been proposed in the literature to characterise textures (Mirmehdi et al. 2008). The simplest region-based approach consists of considering the histogram of the image as in Fig. 5.3. This shows the binarisation into regions based upon a grey-level histogram; the generalisation of this technique to colour histograms (e.g. using three dimensions for red, green and blue) is easy to imagine.

To take the idea of regions further into the texture domain, we present a few other descriptors, for a region R including N pixels in a grey-level image $I(x,y)$:

- The moments of degree n: $m_n = \frac{1}{N}\sum_R I^n(x,y)$ where m_1 is the average grey-level value on R, or the centred moments of degree n, $\mu_n = \frac{1}{N}\sum_R [I(x,y) - m_1]^n$ where μ^2 is the variance of the grey-level values on R and $\sqrt{\mu_2}$ the standard deviation of the grey levels on R. Broadly speaking, the first moment is the mean intensity; the second moment is the variance (which measures how similar the region's intensities are); the third moment is known as *skew*, which describes how symmetrically distributed the values are about the mean intensity value; and higher-order moments are harder to describe intuitively but still capture some characteristics of the region's texture.
- The autocorrelation function $C\left(\vec{t}\right) = \frac{1}{N}\sum_R I\left(\vec{r}\right) I\left(\vec{r} + \vec{t}\right)$ where $r = (x,y)$ and $\vec{r} + \vec{t}$ belong to R, being a translation vector. Autocorrelation functions are a measure which can detect repetitive texture patterns.
- The autocovariance function $\Gamma\left(\vec{t}\right) = \frac{1}{N}\sum_R \left[I\left(\vec{r}\right) - m_1\right]\left[I\left(\vec{r} + \vec{t}\right) - m_1\right]$ where $\vec{r} + \vec{t}$ belongs to R; this is in a sense an un-normalised version of the autocorrelation.
- The power spectrum $S(u,v) = |TF[I(x,y)]|^2$.
- Fractal parameters: for the pixels in R, one counts the average number $N(r)$ of pixels closer than a distance r and satisfying a given criteria. On can, for instance, consider the criterion $I(x, y)$, grey-level intensity greater than a given threshold. A fractal signature is recorded when the representation of $\log(N(r))$ as a function of $\log(r)$ shows a linear behaviour with a non-integer slope. An integer slope of 1, 2 or 3 corresponds to a repartition of the valid pixels homogeneously positioned on a mono-, bi- or three-dimensional curve.

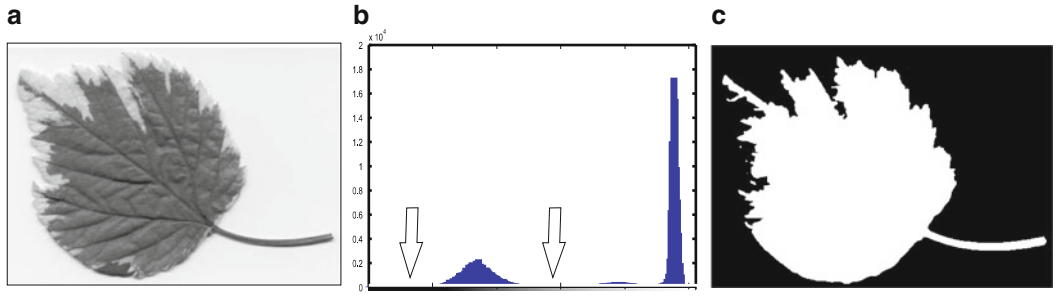

Fig. 5.3 (**a**) An image of leaf; (**b**) histogram of image (**a**); (**c**) binarised version of (**a**) with pixels set to 1 for grey levels between the two *arrows* in (**b**) and pixels set to 0 elsewhere

– The co-occurrence matrix: for a translation $\vec{t}$ for all pairs of intensity $(a,b) \in 0,1,2,\ldots,(L-1)^2$, L being the number of possible grey levels, the co-occurrence matrix is the table containing

$$M_{\vec{t}(a,b)} = card\left\{\left(\vec{r},\vec{r}+\vec{t}\right) \in R \times R \middle| I\left(\vec{r}\right) = a; I\left(\vec{r}+\vec{t}\right) = b\right\}.$$

Often, to limit the computations associated with the construction of the co-occurrence matrix, the images are requantised on $L=8$ or 16. One can then extract numerical features from this co-occurrence matrix such as the homogeneity H

$$H = \frac{1}{N_c^2}\sum_{a=0}^{L-1}\sum_{b=0}^{L-1}\left[M_{\vec{t}(a,b)}\right]^2$$

where, N_c is the number of couples or also the contrast C

$$C = \frac{1}{N_c(L-1)}\sum_{k=0}^{L-1} k^2 \sum_{|a-b|=k} M_{\vec{t}(a,b)}$$

or the directivity

$$D = \frac{1}{N_c}\sum_{a=0}^{L-1} M_{\vec{t}(a,a)}.$$

Grey-level co-occurrence matrices are a very popular texture representation, which can capture details of texture that are difficult to represent with simpler, more parametric measures.

The segmentation of an image into homogeneous regions is made by applying some criterion of homogeneity on a domain R based on the numerical value of attributes like the ones presented above. If these attributes share the same value up to a given tolerance on R, then the region is decided to be homogeneous. Two approaches can be followed:

– Segmentation by fusion: the image is explored from the most basic homogeneous regions, i.e. the pixel. Two adjacent pixels are associated if they satisfy the homogeneity criterion. This approach can be unsupervised or it can be supervised by the manual selection of pixels located in the regions to be extracted. This is called region growing.
– Segmentation by separation: in this case we start with the entire image. If the homogeneity criterion is not satisfied, the image is divided in four subregions (quadtree) and the process is iterated in the subregion up to the stabilisation of the criterion of homogeneity.

Texture attributes can be defined on grey-level intensities or on colour. The recent introduction of depth imaging at a low cost provides access to range intensities (Biskup et al. 2007; Omasa et al. 2007; Klose et al. 2009; Kraft et al. 2010; Fiorani et al. 2012; Chene et al. 2012).

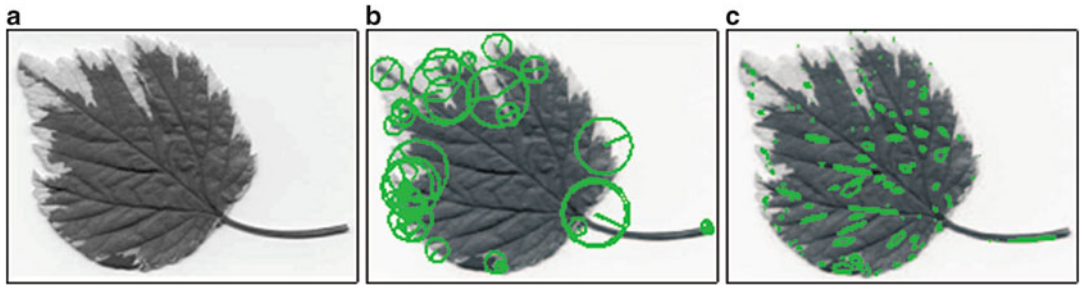

Fig. 5.4 (**a**) An image of leaf; (**b**) in green keypoints extracted from Lowe's SIFT or "scale invariant feature transform" (Lowe 2004); (**c**) the same leaf but with maximally stable extremal regions (Matas et al. 2002) *highlighted*

Information from depth can be of great value when characterising structures like the shoots of plants, which can be more textured in 3D than in grey-level intensity or colour. The adaptation of the state-of-the-art attributes of texture, presented in this chapter, to depth maps when applied to plants constitutes a research topic of current interest.

5.3.3 Keypoints

Keypoints, also known as interest points, are a more recent concept in computer vision. Instead of describing the entire object, these extract only points which have a specific local environment or *image neighbourhood*. This can be expressed in terms of local edges or local texture. The recognition of an object is then based on the detection of a set of keypoints, with a low sensitivity to a partial occlusion of the object as long as a subset of the keypoints is still observed. A variety of keypoint detectors have been developed in the literature including corner features, blobs, ridges, edges and more (see Schmid and Mikolajczyk 2005 for a comprehensive comparison and evaluation). These keypoints have not yet seen with much use in plant imaging. However, there could be room for the development of specific environment defining keypoints of interest for plants like nodes in a branching structure and local orientation on a leaf. Figure 5.4 shows an illustration of the type of output produced by two different keypoint extraction methods.

5.4 Open Challenges for Computer Vision in Plant Sciences

The elements of imaging and image processing presented in the previous sections are standard approaches. They have been applied with success in other domains of applied computer vision, such as industrial vision and biomedical imaging. We believe, therefore, they constitute a good basis for the design of plant phenotyping systems. However, there are some properties of plants, encountered rarely in industrial vision and biomedical imaging, which make imaging for plant phenotyping a specific field of application for computer vision. We propose to discuss in this section those areas we consider as open challenges for imaging and computer vision in plant sciences with optical technologies.

5.4.1 Growth

The longitudinal follow-up of leaving organisms during their development is an important issue in life sciences (see Spalding and Miller 2013 for a recent review on the link with image analysis). The very early development is morphogenesis, i.e. the process during which the forms and shapes of the organism change continuously over time to reach a functional adult stage. Imaging this process in plant science is easier than in the biomedical domain for multiple reasons, including ethics, the absence of movement of the embryo in plants and the possibility of

constituting larger cohorts monitored at high sampling rates.

An open challenge in plant imaging, of utmost importance to the characterisation of plant morphogenesis, is the 3D quantitative monitoring of growth of an entire plant at a cellular resolution. This is possible for young plants at the seedling stage, for which there exists imaging techniques, such as X-ray tomography (Wells et al. 2012) or confocal microscopy (Fernandez et al. 2010), capable of giving access to the entire seedling at a cellular resolution. However, these techniques have some intrinsic limitations with the use of contrast agent and 3D aberrations in confocal microscopy or the relatively high cost of microtomography and possible mutagenesis of the early stages of plant development due to X-ray radiation. However, the recent introduction of new sources of light (synchrotron, femtosecond lasers, etc.) and the progress in the domain of biomarkers and in the performances of sensors (GaAs, CMOS, etc.) are triggering a huge amount of development in microscopy (Anonymous 2012). Taking advantage of these advances, other imaging techniques compatible with the acquisition of entire seedlings at a cellular resolution are emerging tools producing new data to be processed for plant image analysis (see, e.g. Sena et al. 2011 for light sheet microscopy, Cloetens et al. 2006 for synchrotron imaging or Lee et al. 2006 for optical projection tomography).

In addition to the consideration of the cellular level, growth implies morphological changes in the plant domain which simply do not happen in many other arenas: if one were to try and model the growth of an animal, it would be necessary to deal with the shape change, but only within certain parameters. Unlike plants, which develop more leaves and change shape entirely, most other growing organisms keep the same broad architecture. Modelling the growth of baby humans, for example, does not require one to take into account the development of extra legs in the same way that young plants require the modelling of the appearance of new leaves. The regularity of architecture has been exploited within vision, for example, when detecting flowers (Nilsback and Zisserman 2010). Thus it is not only growth at the microscopic level that is of interest. And once one is modelling growth at more than one scale, the question arises of whether it is possible to align models derived from imaging at multiple scales (registration).

5.4.2 Occlusion

The shoots of adult plants form complex three-dimensional structures which produce multiple occlusions. As a marker of this complexity, the shoots of plants have been shown to display fractal properties with scale invariance in 2D projections (Ruderman and Bialek 1994; Chene et al. 2013) or in 3D (Boudon et al. 2006). The detection and segmentation of objects (fruit, single leaves, branches, etc.) in these complex scenes constitute challenging tasks for computer vision. The keypoints mentioned in the previous section could constitute interesting tools for such detection and segmentation of self-similar occluded objects. For some other applications, the full reconstruction of the shoot is necessary. The development of cost-effective 3D optical scanners to reconstruct the shoot of the plant is receiving increasing interest (Biskup et al. 2007; Omasa et al. 2007; Klose et al. 2009; Kraft et al. 2010; Fiorani et al. 2012; Chene et al. 2012). Up until now these 3D optical scanners have used existing devices, but there could be interest in designing new sensors optimising the optics, lighting wavelength, especially for plant phenotyping applications such as those recently proposed in van der Heijden et al. (2012) and Billiot et al. (2013). Also for a given 3D optical scanner, there are open questions like the minimum number and the optimal positions of the pose for a 3D reconstruction of the shoot of the plant at a determined spatial resolution. Similar questions exist in robotics within the field of simultaneous localisation and mapping (SLAM), and there could be interest in considering the application, transposition or adaptation of such techniques to the mapping of plants.

5.4.3 Ground Truth and Public Data Sets

The fact that imaging techniques for certain key biological questions are still under discussion demonstrates that imaging for plant phenotyping is still in its infancy. The diversity of plant species together with their various possible observation scales (cell, seed, seedling, meristems, leaves, branching structure, fruit, entire plant, canopy) calls for the design of a variety of machine vision systems dedicated to plant phenotyping. There are however some generic problems in plant science such as the monitoring of growth and the 3D reconstruction of the shoot. Also there are some plant models like *Arabidopsis thaliana* or *Medicago truncatula* which serve as reference in the scientific community of plant scientists. Such generic problems and model organisms help in fixing observation scales and in defining constraints for the design of machine vision.

Efficient practices in terms of light, choice of optics and imaging technology are progressively disseminated with the recent development of a network of phenotyping centres at an international scale (http://www.plantphenomics.com/). If common geometries of machine vision have been developed by distinct research groups for generic problems [e.g. concerning seedling growth, the machine visions discussed earlier are similar in terms of geometry (Subramanian et al. 2013; French et al. 2009; Benoit et al. 2013)], there is a diversity of image processing algorithms (Wang et al. 2009; Kimura and Yamasaki 2003; Subramanian et al. 2013; French et al. 2009) which have been proposed, but the comparison of the performances of these algorithms is still lacking. The validation of image processing algorithms in plant sciences requires comparison with ground truth which can be based on numerical real physical phantoms, annotated images by experts (with recording of expert variability), or numerical simulations of plants. Such ground truth data sets, which are common practice in biomedical engineering, could help in identifying hard problems likely to trigger the interest of computer scientist coming from outside the field of phenomics. Also, since plant phenotyping is likely to produce large data sets, the publication of annotated data sets could constitute a new domain of application for supervised image processing based on machine learning techniques.

When we have large, public, plant phenotyping data sets, the question of evaluation is still not settled. Segmentation evaluation itself is an open-research question; even when considering the relatively simple question of identifying which pixels in an image correspond to a plant and which to background, the judgement of whether one algorithm performs better than another is a difficult question. Measures such as ROC analysis (Fawcett 2006) which deal with measures of true positive rate against false positive rate fail to capture some intuitive characteristics of segmentation quality. For example, do segmentation errors near the object boundary count more or less than segmentation errors at the edge of the image? Moves towards a more nuanced model of goodness of fit for segmentation in turn need to trade-off against the computational complexity of metric calculation, in particular in low-cost low-power systems (Minervini et al. 2013).

5.4.4 Phenotyping with Low-Cost Imaging Devices

Plants develop through a complex interaction of genotype and environment. Working in controlled environments is helpful from the perspective of scientific investigation, which reduces the variability resulting from these interactions. However, to gain impact and to be able to apply the ideas from image-based phenotyping to real-world problems such as agriculture, we need to also consider plant phenotyping under the variability of field conditions. In these situations, the measurement system has to come to the plant. Different instrumentation approaches can be proposed including phenomobile vehicles embedding sensors, air drones, networks of wireless

sensors installed in the field, cheap webcam-based approaches or handy cameras to be carried by the breeder.

The handy camera approach is not the highest throughput but embeds the unvaluable expertise of the breeder in the measurement phase. The development of such systems can be very low cost if one considers the fact that high-resolution and high-sensitivity cameras are embedded in many current smartphones. An optical probe added on a smartphone can turn a simple telephone into a high-performance and low-cost scientific camera. Only simple optical probes need to be developed to produce a new phenotyping handy imaging system. Images acquired by such equipped smartphones can be directly transferred to a server to be processed. Similar approaches have already been successfully tested in various domains including microscopy (Zhu et al. 2011), biomedical engineering (see Ozdalga et al. 2012 for a review and Pamplona et al. 2011 for an impressive 1 dollar camera to diagnose cataracts). Practically all optically based measurement shown useful in agriculture in the literature could be transformed into such an embedded version. These optical measurements could be complemented by GPS localisation as well as environmental information: exploiting, for example, weather information from the Internet services. We believe this is a particularly promising avenue of research, which could develop communities of breeders contributing to plant phenotyping.

5.5 Conclusion

This chapter has presented an overview of computer vision for plant science, highlighting key computational concepts, progress made and open-research questions. For imaging technology to play a full role in the development of phenotyping systems, automated analysis is vital: a large Lemnatec installation, for example, can capture upwards of 4,000 images of 800 plants in a day. Time-lapse photography, photographing plants once per minute, is not a new thing – but cost-effective time-lapse photography with a camera per plant is certainly within reach now. Opening up plants to this level of scrutiny may well expose traits that have never before been measurable. It is easy to understand that the vast majority of these images will never be looked at by a human being, and so without automated measures of plant characteristics, the potential for these systems will never be realised.

Image analysis and computer vision for plant science are not without pitfalls and drawbacks; however, as with any interdisciplinary work, it works best when both parties (biologists and computer scientists) work together on a solution from the outset, considering image capture setup (light, geometry, technology) as well as the specific scientific questions in terms of phenotypic measurement. This is an exciting domain to work in, which should prove fruitful for both computer science and for plant biology, but for real progress, it is important that both disciplines understand each other.

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Screening for Plant Features

6

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Abstract

In this chapter, an overview of different plant features is given, from (sub) cellular to canopy level. A myriad of methods is available to measure these features using image analysis, and often, multiple methods can be used to measure the same feature. Several criteria are listed for choosing a certain (set of) image descriptor(s) to measure a plant feature. The choice is dependent on a variety of reasons, including accuracy, robustness, recording time, throughput, costs and flexibility. We conclude that hyperspectral imaging can provide a powerful set of image descriptors, which can be used to measure numerous plant features using multivariate statistical models. However, care should be taken that the estimates obtained with these statistical models provide the right measurement for the plant feature under all circumstances of interest.

6.1 Introduction

When breeding for new plant varieties, the breeder first creates new genotypes and then selects the best performing ones from the pool of newly generated genotypes. For a well-informed selection, he/she needs to observe a large number of features. The features can be molecular markers (including genetic, protein or metabolomic) or morphological markers including seed/seedling traits (e.g. related to germination and vigour), but most features will probably be based on the plant phenotype. Plant phenotype features can be assessed on individual plants or on a plot basis and range from early vegetative development to a more mature generative stage.

Over the last century, tremendous gains have been achieved in plant breeding. For example, in Kansas, wheat yield increased by 26 % over 26 years due to genetic improvement (Barkley et al. 2013). However, since most "low hanging fruit" has been picked by now, breeders need to make still larger efforts to further gains. For this, they are investing heavily in biotechnology as well as advanced phenotyping techniques.

Recent biotechnological approaches are not only a powerful tool in finding genes and

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generating new genotypes, they are also of great help in selecting genotypes which contain the positive alleles of certain genes. For example, for disease resistance, it is often quite cumbersome to conduct an experiment to establish which genotypes are (slightly) more resistant. By using marker-assisted selection for alleles that are known to have a positive effect, the chances of selecting a genotype that expresses a positive effect are considerably increased. However, in the end, it is always the phenotype that counts and not its genetic marker pattern. Thus, it is important to use advanced phenotyping technologies to face the challenges of modern breeding. Plant phenotyping can be defined as the set of methodologies and protocols used to measure plant growth, architecture and composition with a certain accuracy and precision at different scales of organisation, from organs to canopies (Fiorani and Schurr 2013). The term is generally restricted to plant breeding purposes, and not used for plant production, like sorting. Digital plant phenotyping refers to the use of computers for plant phenotyping where digital sensors are used to measure plant characteristics. One of the most common digital phenotyping methodologies is image analysis, where cameras are used to record images and software is used to automatically extract the measurements from the images, in a reproducible and accurate way (please see Chap. 4 for digital phenotyping).

There are several approaches that can be taken to measure plants using image analysis. The most obvious way is to use it as a digital ruler mimicking hand measurements for specific plant features. This is done in plant variety testing, where the aim is to assess whether a candidate variety is sufficiently distinct from all other varieties of common knowledge in order to grant the plant breeder's rights. The assessment is made on a character (plant feature) by character basis, where the characters are defined and described in international guidelines of UPOV (http://www.upov.int/test_guidelines/). It requires reliable, objective and reproducible measurements of distinctive plant parts. Image analysis has therefore been used in plant variety testing since early 1990s, to automate the measurement process. Examples include the measurement of the size and shape of wheat kernels (Keefe and Draper 1986) and various other plant parts of other crops such as beans and onions (van der Heijden et al. 1996). In plant variety testing, the aim is to have a single image descriptor that serves as a measurement for a well-specified plant feature. Here, an image descriptor is loosely defined as some image property, which is expressed in a digital number using a computer algorithm.

In plant breeding, the purpose is generally aimed at meeting specific breeding targets, e.g. having increased yield under certain stress conditions. Components of breeding target are often necessary since the final target, like yield, is the result of so many interacting genes and varying environmental factors that its heritability is very low. Dissecting the breeding target in several constituent physiological features with a hopefully simpler genetic basis is therefore an interesting strategy (Hammer et al. 2006; Alimi et al. 2013).

One approach to dissect yield in plant parameters is used in crop growth models. Crop growth models aim to increase the understanding of crop behaviour by explaining crop growth and development in terms of the underlying physiological mechanisms (Bouman et al. 1996). The advantage of this approach is that it is an integral approach that would allow the prediction of yield from the underlying components over a range of (untested) environments. The approach is however rather ambitious, as it may involve estimation per genotype of rather difficult to acquire parameters, which can only be extracted from measurements over a range of conditions. Messina et al. (2006) showed that it was possible to develop a gene-based model to simulate soybean development and yield of different genotypes. The crop growth model 'CROPGRO-Soybean' was combined with linear models that predict genotype-specific parameters as functions of six so-called "E" loci. The results suggest that gene-based approaches can effectively use agricultural genomics data for the prediction of genotype performance.

Using molecular markers in combination with automated phenotyping to estimate the most important parameters in order to calibrate the crop growth model for individual genotypes is a very interesting approach, which certainly requires further research, although it is not an easy task. Another less ambitious approach is to look at a selected set of experiments and treatments (lab assays, greenhouse screens or field trials) and measures only those plant features that capture the most important aspects of the breeding target in these experiments and heavily automate the phenotyping for these features using sensors like cameras.

This chapter aims to give an overview of various relevant plant features and corresponding image descriptors that can be used to estimate the features using digital plant phenotyping.

6.2 Criteria for Measuring Plant Features

For measuring plant features using digital imaging, several criteria have to be taken into consideration depending on the type of feature. A non-exhaustive list of criteria is:

- What is the scale at which we want to measure? Is the plant feature expressed at a microscopic scale (at or below the level of cells) or a macroscopic scale (examining different plant parts), or is it measured at the total plant level (e.g. total leaf area) or even at the crop/plot level (e.g. yield)? For microscopic images, we generally work at a more destructive level and at a low-throughput rate (although some processes can be automated). Because of the limitations in throughput, this scale is not frequently used as a standard phenotyping method, but mainly for specific research purposes. It can provide interesting information, e.g. on cell size, cell wall thickness or specific chromosome arrangement. The macroscopic and plant scales are the most frequently used scales, and a large variety of methods exist, which will be discussed in more detail later in this chapter. Sensing at the crop/plot level can be done in the field with manually operated platforms. Of late, unmanned ground or aerial vehicles (UGV and UAV, respectively) also offer great potential and are currently the topic of research (Perry et al. 2012). One can also use remote cameras in aeroplanes or even satellites, but generally, they have a limited resolution and can only give information on crop level during the season. This may be a limitation for breeding purposes. Further, the possible disturbances and fluctuations of atmospheric conditions and, for satellites, the strict intervals of recording may cause limitations on their applicability.
- Some plant features may be better discernible under specific illumination conditions with special sensors. For example, anthocyanins can be observed in the visible part of the electromagnetic spectrum, but may be more reliably measurable in the ultraviolet part of the spectrum. Other compounds like fatty acids are better assessed in the (near) infrared part of the electromagnetic spectrum. The plant response to stress can be measured with thermal imaging cameras or by using special light and cameras using the fluorescence property of chlorophyll (Pieruschka and Poorter 2012). Hyperspectral imaging offers great potential to measure water content or specific compounds like carotenoids (Polder et al. 2004).
- Closely related to this is the question whether the feature is visible from the outside of the plant (reflectance imaging can be used) or if it requires information from within the plant. In the latter case, we may have to revert to transmission imaging techniques like X-ray or nuclear magnetic resonance (NMR; please see Chap. 12 for more details).
- Can the features be recorded on individual plants, or are the plants standing in plots, overlapping and intertwining with other plants? If plants are in individual pots, they can be put on conveyer belts and transported to a special recording cabinet, which are equipped with specific cameras and lighting

arrangements and, e.g. turntables, allowing detailed and controllable phenotyping. If plants cannot be individualised but are standing in plots, either in a growth chamber, greenhouse or in the field, it generally becomes more complicated. For example, the illumination is less well controlled, the plants may be intertwined, and plants cannot be easily imaged from all sides, which increase the probability of occlusion and hamper the possibility of 3D imaging. On the other hand, imaging the plants in plots has advantages as it is more closely related to their standard growing environment.

- Can the trait of interest be measured with image descriptors in a 2D image, or is 3D information required? In many situations, the size and shape of 3D objects can reliably be measured in 2D, and these features are much easier automated. But in some cases, 3D information is required, e.g. when the scene is more complex and contains different objects at different distances from the camera. A variety of 3D camera systems are entering the market, offering great potential to automate more complex measurements.
- Is the image descriptor a direct measurement of a plant feature, is it a proxy for a certain feature or is it otherwise useful in its own right? The image descriptor is a direct measurement if, for example, we measure the length of a seed by means of image analysis in the same way as we would do manually by a ruler. In this case, the aim is to have the same results with both the manual and automated methods (after proper calibration), irrespective of the phenotypic expression.
- Is the plant feature observable above ground (e.g. leaf length or number of stems) or below ground (e.g. root length)? This is a straightforward distinction, and it is important as in general, it is less complicated to examine plant parts above ground than below ground. In some cases, below-ground observations can be made easier by growing plants on special systems, such as hydroponic systems or even transparent soils (Downie et al. 2012). This has influence on the plant root system in comparison to its default growing conditions, and it should be verified how the features in this system translate to the normal growing conditions. We refer to Chap. 8 for a more detailed description of underground features especially root traits.

Image descriptors are closely linked to the method of recording. The recording method poses restrictions on the time needed for a recording (it can range from milliseconds for a normal colour image to several minutes for nuclear magnetic resonance or for certain photosynthesis parameters), the level of automation (conveyer belt systems, robots or satellites), the level of control of the environment (e.g. active or passive illumination) and the operating costs. These aspects are not further considered in this chapter but have been discussed elsewhere in this book.

Once the images are recorded, many image descriptors can be measured quickly and reliably without any human operator, which is a requirement for high-throughput phenotyping. In some instances, we can allow a certain level of user interaction to verify and adjust the image analysis step. Finally, in some instances, e.g. during the development phase, descriptors in the image are totally measured by a human being, where the user has to indicate by a mouse or other pointing devices the feature to be measured.

In Table 6.1, an overview of various plant features is given with a list of image descriptors and other criteria. The list is not meant as an exhaustive list, but only as an indication of the various approaches available to measure certain plant features.

6.3 Assessing the Performance of Image Descriptors

6.3.1 Comparison with Manual Measurements

In many situations, image descriptors are aimed to replace manual measurements of plant features. The standard approach is to make a direct comparison between the image descriptor

Table 6.1 An overview of various image descriptors that can be used to measure plant features at different scales and in different environments

Scale	Environment: field, greenhouse and lab	Plant parts/ target trait	Plant feature	Measurement method	Image descriptor	Wavelengths	References
Microscopic	L	Plant part constituents	Chlorophyll, flavonoids and carotenes	Multispectral fluorescence	Area (nr of pixels) of linear discriminant analysis	UV, VIS, NIR	Baldwin et al. (1997)
Microscopic, macroscopic	L	Plant part constituents	Lignin	NIRS	PLS model of spectrum	NIR	Hatfield and Fukushima (2005)
Macroscopic	F, G, L	Leaves/fruit constituents	Chlorophyll concentration	SPAD	Absorbance index	VIS, NIR	Rorie et al. (2011)
Macroscopic	F, G, L	Leaves/fruit constituents	Chlorophyll, flavonoid and carotene concentration	Hyperspectral imaging	PLS model of spectrum, averaged over region	VIS	Polder et al. (2004)
Macroscopic	F, G, L	Leaf photosynthesis	Photosystem II	Fluorescence	NPQ, Fv/Fm	Excitation: blue/red Emission: 720 nm	Barbagallo et al. (2003)
Macroscopic	F, G, L	Leaf stress	Stress: closed stomata reduce transpiration leading to increased temperature	Thermal imaging	Local temperature	IR	Merlot et al. (2002)
Macroscopic	L	Seed diseases	Aflatoxin in corn ears	Fluorescence imaging spectroscopy	Pixel count after classification	Excitation: 365 nm Emission: VIS/NIR	Hruska et al. (2013)
Macroscopic	F	Leaf diseases	Southern leaf blight in corn leaves	RGB imaging	Pixel count after classification	VIS	Green et al. (2012)
Macroscopic	F	Leaf diseases	Virus in tulip leaves	Multispectral imaging	Pixel count after classification	VIS	Polder et al. (2010, 2013)
Macroscopic	L	Organ dimensions	Size and shape of seed	B/W imaging	Specific algorithms	VIS	Keefe and Draper (1986)
Macroscopic	L	Organ dimensions	Size and shape of range of plant organs	B/W or RGB imaging	Specific algorithms	VIS	van der Heijden (1995)
Macroscopic	G	Organ dimensions	Size and shape of leaf of pepper plants	3D imaging	Specific algorithms	VIS	van der Heijden et al. (2012)
Plant	G, L	Plant architecture	Root architecture	X-ray, NMR and many more	Diverse	Diverse	Fang et al. (2012)

(continued)

Table 6.1 (continued)

Scale	Environment: field, greenhouse and lab	Plant parts/ target trait	Plant feature	Measurement method	Image descriptor	Wavelengths	References
Plant	L	Plant architecture	Above-ground architecture	3D methods	Diverse	Mostly VIS	van der Heijden et al. (2007)
Plant	F	Leaf water relations	Water content	Hyperspectral	PLS model	VIS/NIR	Schut et al. (2006)
Plot							
Plot	F	Plant part constituents	Sugar and lipid concentration	Hyperspectral	Hyperspectral signal	VIS/NIR	Schut et al. (2006)
Plot	F	Plant part constituents	Nitrogen	Hyperspectral	PLS model of spectrum	VIS/NIR	Schut et al. (2006)

and the hand measurements over the range of expression (different genotypes) of the plant feature of interest. The hand measurements are then used as ground-truth data, and the correspondence can be modelled with a regression model. A simple regression model can correct for offset and slope, but more complicated models can be used to model a curvilinear relation over the range of expression. Another aspect that is often overlooked is the error in the hand measurements by the human expert. There may be some variations for repeated measurements even by the same expert, as well as variation between measurements of the same object by different crop experts. When building the ground truth, these aspects need to be taken into account.

Often, the approach of one image descriptor for one plant feature requires rather precise image segmentation, dividing the image in relevant clusters (objects and background). If proper illumination can be provided under standardised conditions, segmentation can be as simple as a threshold value, where pixels in the image below the threshold are assumed to be foreground pixels (belonging to the object of interest) and the others background (or vice versa). If the imaged scene is more complex, segmentation can be rather cumbersome. A range of more or less sophisticated segmentation algorithms exist (Gonzales and Woods 1993), as there is no single segmentation algorithm that works under all circumstances. Each algorithm has its assumptions, and if these are not met in practice, the effect is often not predictable. If conditions allow for simple thresholding, this is preferred, due to its simplicity and robustness. Therefore it is generally worthwhile to invest time in trying to optimise the image recording, to allow for easy and robust (threshold-like) segmentation.

If the object (e.g. leaf or fruit) can be segmented in the 2D image, many descriptors of this object can be extracted from the image, such as perimeter length, Feret diameters, area, roundness, eccentricity, the average and standard deviation of the grey value or colour (Gonzales and Woods 1993). It should be noted that even a correct segmentation of the object in the image may yield descriptors which do not provide a good measurement for the plant feature of interest, as generally the original object is a 3D object and only a 2D projection of the object is available in the image. For example, the measured projected leaf area is strongly dependent on the angle of the leaf with respect to the camera. Thus the feature must be measurable from the projection in the 2D image. If this is not the case, one may try to use information from 3D images or other statistical approaches. In any case, the image needs to be adequately calibrated to establish the relation between the pixel size and the real world dimensions.

6.3.2 Direct or Indirect Measure of a Plant Feature

It can be difficult and tedious to develop an algorithm for an image descriptor to properly measure the feature of interest. Therefore, in many cases, one will not try to develop an image descriptor that exactly mimics the manual measurement process. Often, a suitable approach is to develop an image descriptor that serves as a proxy for the feature, showing a good correlation over the range of expression in the set of genotypes. This correlation has to be tested from time to time, especially if new genotypes are introduced which may have an expression of the feature, out of the tested range.

If the translation of an image descriptor to a plant feature needs calibration for each specific genotype, it is not really suitable for breeding purposes as is discussed for leaf area (Gao et al. 2012). An example of a proxy is the measurement of leaf area index or total leaf area, simply by counting the number of green pixels in an image. This count is not the same as the area of all individual leaves, but it generally shows good correlation (van der Heijden et al. 2012).

It may be hard to find a single image descriptor to estimate the feature of interest in plants. In that case, one may still be able to estimate the plant feature by measuring a collection of image descriptors, like statistics over a certain image region (mean, standard deviation, histogram

distribution, etc.), and use these in a statistical model. An example of such a feature and its corresponding set of image descriptors is the estimation of total leaf area using colour histograms by means of principal components regression. Another example is the use of hyperspectral data to measure concentrations of plant compounds like chlorophyll or carotenes in a spatial preserving way (Polder et al. 2004). A large toolkit of statistical models or machine learning tools is available to combine a set of image descriptors to estimate a plant feature, such as multiple linear regression, Lasso, partial least squares, neural network, support vector machine and regression trees (Hastie et al. 2009).

In the above cases, the criterion for an image descriptor is the correlation with a plant feature, but one of the powers of image analysis is to use the computer to generate descriptors for plant features that we cannot easily assess or quantify ourselves, but which still bear useful information. An example is the measurement of texture, which can be quantified by a set of image descriptors like Gabor features (Gonzales and Woods 1993).

6.3.3 Criteria for an Image Descriptor

A criterion for an image descriptor is its accuracy, i.e. the image descriptor should correspond with the plant feature of interest in such a way that there are no systematic differences (bias) for different genotypes. A nice example of bias is the height of onion bulbs (van der Heijden et al. 1996). The computer-determined height corresponded well with the hand measurement for many genotypes, but not for genotypes with a broad neck. This is due to another definition of the top of the bulb by the computer (at the inflection point), compared with the visually assessed top. If it is acceptable that a new definition of bulb height is used and that there is a possible break with traditional manual measurements, the breeder can apply this new definition and in the future rely on the objective measurement of the image descriptor. If not, one can include extra terms in the calibration model, for example, the neck thickness, to try to correct for the bias.

Another example is to use projected leaf area instead of leaf area index or total biomass. Clearly, projected leaf area is not the same as total leaf area, but, depending on the application, it might be sufficient. This can be tested in a validation experiment with a representative sample of the genotypic variation, sufficiently covering the range of expression of the feature.

Especially with correlative models, we have to be aware of their limitations. For example, the projected area and length of an ear are all highly correlated, so one can simply use projected area instead of ear length. But it might be that we are more interested in genotypes with ears having the same projected area, but a shorter length. In this case, it is trivial to measure both features, but in the case of hyperspectral imaging, such relations are much more complex. Therefore, care should be taken for holding the relationship between the plant feature of interest and the image descriptors.

Besides accuracy, an image descriptor should also be precise (low variation) and reproducible (same value if the measurement is repeated). For example, van der Heijden et al. (1996) compared the variation of a new image analysis method with that of the existing (visual/manual) method by making multiple (independent) measurements of the same object and looked at the means and standard deviation for the different genotypes. The means should be on a straight line $y=x$, and the standard deviations should preferably be lower for image analysis than for hand measurements.

A third criterion is the heritability of the image descriptor. Heritability is a measure for the fraction of phenotype variability that can be attributed to genetic variation. If the genetic variation (differences between the genotypes) is high compared to the other sources of variation, then the feature offers good possibilities for selection. A simple method, rather equivalent to heritability, is to look at the F-ratio statistic of the mean squared error of genotypes over the mean squared error of samples in an analysis of

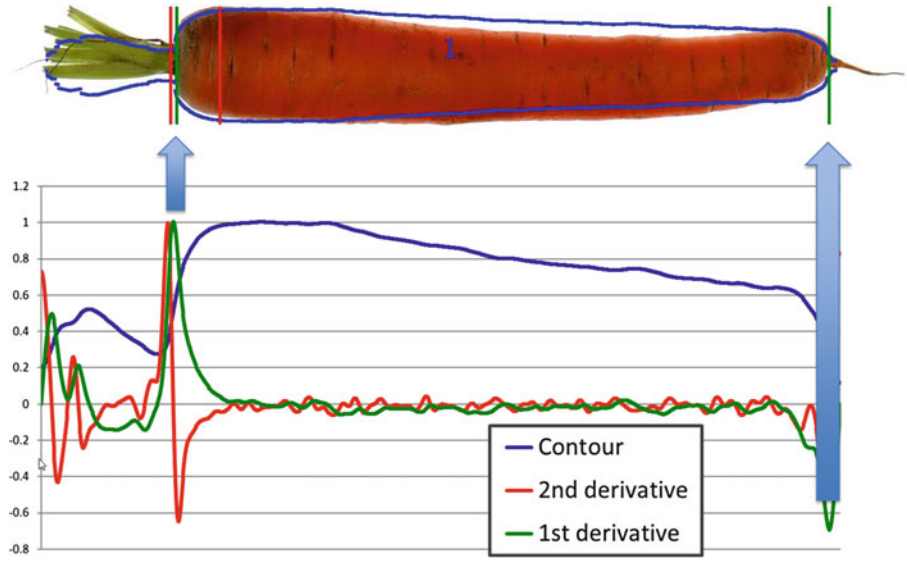

Fig. 6.1 The shape of elongated objects like carrots can be described using the local width as a function of the length axis. First- and second-order derivatives of the width array can be used to locate specific points, like the start and end of the neck

variance. If this F-ratio is highly significant, this is an indication that the heritability is high.

Another criterion for an image descriptor is to examine whether quantitative trait loci (QTL) can be found for the descriptor. A QTL is a segment of DNA that contains or is linked to genes that underlie a quantitative trait. If we have a population of genotypes, either a broad (association) panel of diverse genotypes with different backgrounds or an offspring of a crossing between two homozygous inbred lines (i.e. a population of recombinant inbred lines), we can look at associations between molecular markers on the genome and the descriptor. If we find high correlations, it indicates that the descriptor has some genetic basis that can be used in marker-assisted selection. This approach was used by van der Heijden et al. (2012), where they were able to find the same QTLs for several image descriptors and for their corresponding plant features, like leaf size, total leaf area and plant height.

6.3.4 Composite Plant Features

Many plant features are composite features that cannot be measured with a single descriptor. For example, the shape of a leaf is a feature that cannot be captured on a unidirectional scale, and it requires multiple descriptors for the different aspects of shape. Also, the colour of a leaf is not just a single value or even the simple combination of the three colour components red, green and blue (or another colour transformation). Since colour can vary over the leaf, one can have average colour, main colour, secondary colours as well as colour variation, colour patterns, etc. In general, a set of image descriptors is then required to capture the plant feature or an aspect of it.

An example of a combination of image descriptors that provide shape information that is difficult to quantify in plant features is the shape of elongated, symmetrical objects like carrots. The shape of a carrot can be described using an array of width values as a function of the relative position along the length axis (Fig. 6.1). By averaging this width array over multiple carrots of the same plot, the average width as a function of its position along the length axis can be obtained per genotype, hence creating a visual representation of the average carrot of that plot. An example is shown in Fig. 6.2. This local width array per plot can be compared with the average

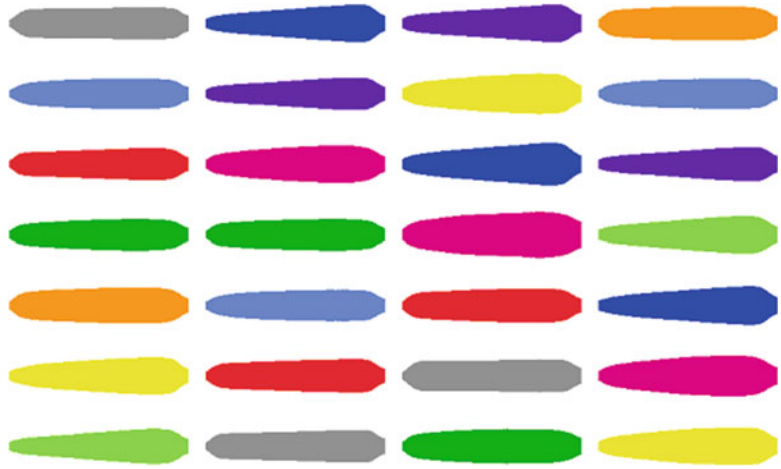

Fig. 6.2 The local width array can be averaged over multiple carrots of a plot or genotype, hence creating a visual representation of the average shape of a plot/genotype. Carrots with the same colour represent different plots (replications) of the same genotype

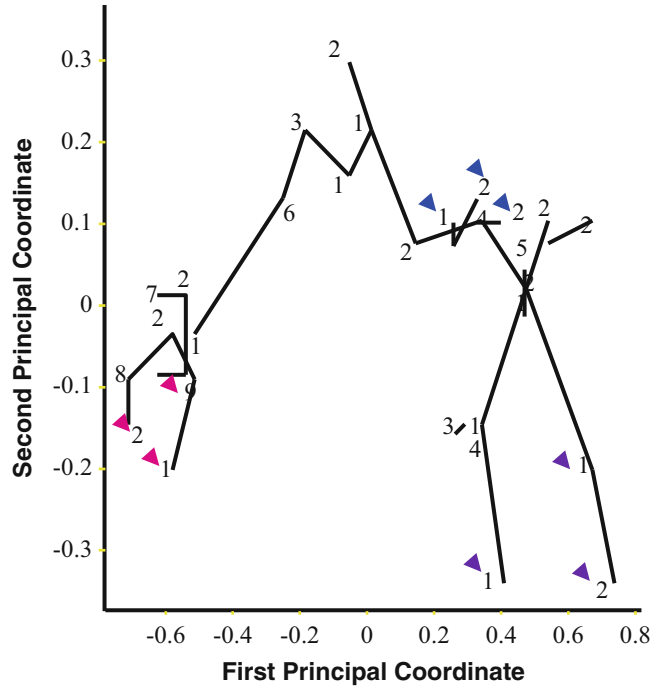

Fig. 6.3 A multivariate distance (in this case, Euclidean) can be calculated between every pair of average carrot shape descriptions, and these distances can be shown in a two-dimensional graph using multidimensional scaling techniques like principal coordinates analysis. Points that are closest together in the multidimensional space are connected together as a so-called minimum spanning tree. The *colour markers* next to points refer to the same colour in Fig. 6.2. The same colour indicates multiple plots (replications) of the same genotype

array of another plot by calculating a shape distance index between the plots, e.g. by using a Euclidean distance, which is the square root of the squared differences over the entire length axis. The thus obtained distance matrix for each pair can be analysed using multidimensional scaling methods like principal coordinates analysis to visualise the (dis)similarity of genotypes in a multivariate sense (Fig. 6.3). Combined with the length of the carrot, it gives a rather complete information-preserving impression of the average size and shape of a genotype. Another example to describe the shape of a 2D object in an information-

preserving way is by Fourier descriptors or curvature along the contour (van Otterloo 1991).

Often, it will not be possible or necessary to develop an information-preserving description of the shape, texture or colour of an object. In that case, a set of descriptors that together capture the most important aspects of the composite feature can be used. For example, the shape of the carrot could be reduced to a few shape descriptors, like overall average width, maximum width, position of the maximum width along the length axis, and the width near the bottom (5 % from the low end) and top (5 % from the top), and their difference which can provide an indication if the carrot is tapered or cone like. These descriptors for certain aspects of shape can be more useful than the complete shape description, since it is concise and each descriptor can be used in its own right. Of course, the complete (information-preserving) shape description can be used to extract these parameters and other ones, if needed. Therefore, it is advisable to store a complete object description for later use. The easiest information-preserving description of the object is the original image.

6.3.5 Exact or Statistical Descriptors

If the recording conditions can be well controlled, often a suitable projection of the object can be obtained in the 2D image such that it is possible to find an image descriptor which corresponds well to the feature of interest, e.g. the area of a leaf.

If we cannot fully control the recordings, and the distance between camera and object varies, or the orientation of the object with respect to the camera is unknown, it is more difficult to get a good estimate of the feature. Ideally, the image should contain information required to calibrate the object, like the orientation of the leaf and its distance to the camera to measure leaf area. This information may be obtained using 3D imaging techniques. A wide variety of 3D techniques for plant imaging exists, including stereo-based imaging, laser triangulation, volumetric intersection, structured light, time-of-flight and light-field technology, each with their own strengths and weaknesses. By using a 3D recording technique, it may be possible to segment the leaf in the 3D image, calculate its orientation and magnification and measure its surface correctly. van der Heijden et al. (2012) used the combination of a time-of-flight and stereo approach to create 3D images of pepper plants and automatically extract the leaf surface for plant phenotyping. The rather complex 3D reconstruction approach was needed to correct for distance and orientation of the leaves. Paulus et al. (2013) used a laser-scanning method to measure the ear volume of wheat. Fang et al. (2012) have given an overview of different systems to quantify the 3D root architecture in situ.

There are limitations to a 3D approach also. When the plant structure is complex and the plant has a large number of leaves, it will be hard to discern the full 3D structure with all individual stems and leaves with most 3D imaging techniques, due to occlusion. Exceptions are transmission methods like NMR and X-ray computer tomography, but they are expensive, slow and complex, and generally not well suited for reasonable throughput plant phenotyping. Therefore, generally the 3D method works best with plants that are reasonably open or if the features can be reliably assessed at plant parts not hampered by occlusion.

If a 3D model is too complex, a statistics-based approach can be a good solution. For example, total leaf area could not be measured in the 3D reconstruction of a crop of pepper as the leaves were overlapping occluding each other. By using a multivariate method (principal components regression) based on colour histograms, it was possible to estimate total leaf area (van der Heijden et al. 2012). The QTLs found were similar to those found by a time-consuming and destructive manual measurement of total leaf area.

6.4 Hyperspectral Measurements

Hyperspectral imaging can be used to measure many of the features listed in Table 6.1. Clearly, it is one of the most powerful imaging methods

for plant phenotyping. In hyperspectral imaging, every single pixel consists of an array of values, corresponding with the reflectance, emission or transmission at a certain wavelength. Since a spectrum is available per pixel (Fig. 6.4), hyperspectral imaging not only allows for standard measurements of objects regarding size and shape, but it also offers more information for correct segmentation of the object as well as the application of statistical models like partial least squares to measure plant features like dry matter content, nitrogen status or sugar concentration (Schut et al. 2006), or concentration of lycopene or chlorophyll (Polder et al. 2004). Two approaches are often used to process the hyperspectral images. One is using multivariate methods like partial least squares to build statistical models to map the hyperspectral data to concentration data. The other method is to use the ratio of two or three specific bands to construct an index. PLS-based models can outperform index-based methods as more information can be used for the calibration (Polder et al. 2010). However, for such multivariate methods, the set of images used for the calibration of the model is very critical, and care should be taken to use a sufficient large set of images and genotypes to build the model; otherwise poor and unreliable prediction results may be obtained.

Disadvantages of hyperspectral imaging are the amount of light needed for a good recording, the time needed to acquire a full-spectral image and the amount of data generated and processing power needed to process the large images as well as the possible complexity of combining multiple images together (especially if plants are moving).

Instead of using the full spectrum, one can use a selected number of wavelength bands. Cameras exist that can acquire three to nine bands using special filters in real time. This technique is known as multispectral imaging. A best-of-both-worlds approach may be to use hyperspectral imaging in the research and development phase to do the band selection, while the selected wavelengths are subsequently implemented in a multispectral camera, resulting in fast acquisition and processing for obtaining meaningful plant features for high-throughput phenotyping. Strictly speaking, a colour RGB camera is a form of a three-band multispectral camera, where the sensitivity of the three filters is tailored to match the human eye. Four-band multispectral images with an NIR band added to the

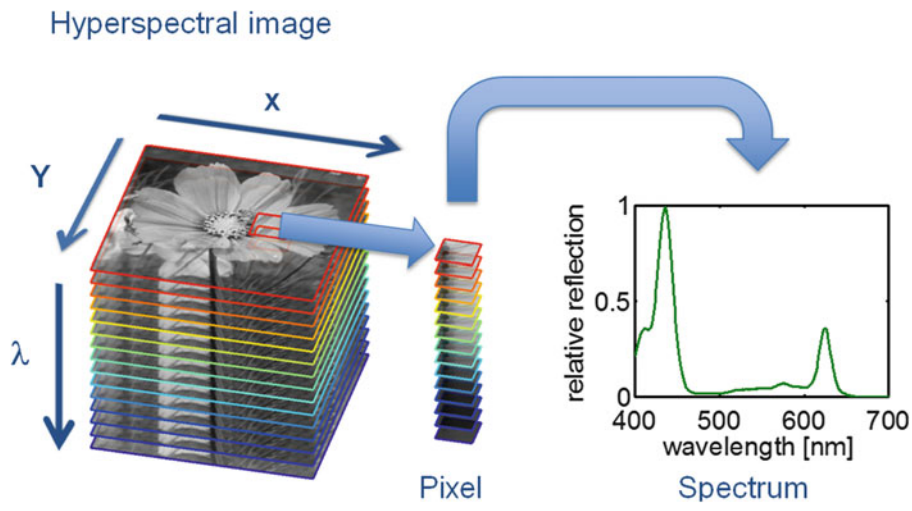

Fig. 6.4 A hyperspectral image consists of a 2D spatial image with a third spectral axis representing the wavelengths of a part of the electromagnetic spectrum, e.g. the visible part (400–700 nm) or the infrared part (700–2,300 nm). Each pixel in the image thus contains information over the spectrum recorded. This spectral dimension can be used to extract multiple features, like concentrations of compounds

RGB bands are used for detection of the Tulip breaking virus (TBV) in tulip fields (Polder et al. 2013).

6.5 Conclusions

In this chapter, an overview of different plant features and image descriptors to automatically assess these features has been given. A multitude of methods is available and there are multiple ways to measure the same feature. In general, the choice is dependent on a variety of reasons, including accuracy, robustness, recording time, throughput, costs and flexibility. Correlative measurements based on composite descriptors, such as can be obtained with hyperspectral imaging, are very powerful, but care should be taken that the estimates obtained with multivariate statistical methods provide the right measurement for the plant feature under all relevant circumstances.

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Phenotyping Crop Plants for Drought and Heat-Related Traits

7

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Abstract

Frequent drought and heat spells especially after the onset of flowering are common events causing substantial yield losses to agricultural production. Researchers lay emphasis on improving the yield potential and stability of crop genotypes by incorporating genes/QTLs for drought and heat tolerance through large-scale phenotyping of germplasm, segregating populations, and elite lines using conventional and modern phenotyping tools. Field screening at different moisture and temperature regimes provides an empirical way of evaluating germplasm and elite progenies for drought and heat tolerance at desired crop stage. The methodologies currently in use for screening germplasm/improved materials for tolerance to heat stress include delayed sowing with supplemental irrigation to let the flowering period of the crops coincide with the period of high-temperature shocks, while for drought tolerance, late planting on receding soil moisture and at low rainfall sites is commonly adopted. In the recent past, modern tools and techniques have been developed for precision phenotyping of drought and heat-related traits. Recent advances in imaging technologies have allowed the estimation of biomass and growth parameters nondestructively and rapidly. This includes easy-to-use tools of spectral reflectance, digital imagery, thermal imagery, and stable isotopes which have been instrumental for large-scale phenotyping of morphological and physiological traits in crop plants. In this chapter, we discuss various phenotyping methods available for breeding climate-smart varieties for adaptation to drought and heat stress conditions.

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7.1 Introduction

Drought and heat are the two important abiotic factors limiting crop yield in rainfed ecology. With climate change, these stresses are predicted to be extreme and frequent. The current trends in climate change have already begun to adversely influence the world agriculture (Lobell et al. 2011). Global temperature is rising steadily, which is likely to have severe impact on the growth and development of crops, especially those grown in tropical and subtropical conditions (Hatfield et al. 2011). It is predicted that the annual maximum temperature will increase by about 1–3 °C by 2050 (IPCC 2012). Recent and potential future increases in global temperatures are likely to be associated with impacts on the hydrologic cycle, including changes to precipitation and increases in extreme events such as droughts and heat waves (Gregory et al. 1997; Wetherald and Manabe 1999; Wang 2005; Seneviratne et al. 2006; Sheffield and Wood 2008). Plant response to water deficit depends on the amount and rate of water lost, duration of drought stress, plant variety/species under consideration, developmental stages of the plant, and other environmental variables such as temperature, relative humidity, etc. Adaptation to heat stress is defined as tolerance to temperatures above a threshold level that results in irreversible damage to crop growth and development (Cairns et al. 2012). Many studies have concluded that this threshold is lower for reproductive development than for vegetative growth period.

In general, the earliest growth parameter reacting to water and heat stresses is the photosynthetic activity. Various crops have been evaluated for their response to drought and heat stresses using agronomic, phenological, morphological, and physiological traits (Pressman et al. 2002; Weerakoon et al. 2008; Cottee et al. 2010; Kumar et al. 2012a, b; Kaushal et al. 2013). Drought and heat not only cause phenological changes but also affect floral organs and fasten the grain-filling period. Legumes are highly sensitive to these stresses during the reproductive phase resulting in substantial loss of flowers and pods with consequent grain yield loss. A number of studies have implicated development and function of pollen as the most sensitive stage to heat stress (Sato et al. 2002; Devasirvatham et al. 2012; Li et al. 2012; Kaushal et al. 2013). High temperatures during reproductive development often negatively impact pollen viability and fertility (Hall 2004), floral bud development (Prasad et al. 1999), seed-filling period (Boote et al. 2005), and seed composition (Thomas et al. 2003). Though many studies have been conducted on the terminal drought and heat stresses in crop plants, effect of these stresses at early developmental stages is less well studied. Therefore, how to maintain plant growth and yield under drought and heat stresses remains the major challenge for plant breeders. Though many efforts have been made to improve crop productivity under drought and heat stress, unfortunately, precise phenotyping of germplasm under reliable conditions remains the most limiting factor.

7.2 Traits Imparting Tolerance to Drought and Heat

Traits that help improve the yields in drought- and heat-prone environments are related to either plant survival or reproductive organ viability or yield expression under the stress. In general, breeders study the genotypic variation for whole plant response to the imposed water and heat stress or identify genotypic variation for specific traits associated with tolerance to drought and heat. Recently, emphasis is also

placed on traits associated with water-saving mechanism under nonstressed conditions (Vadez et al. 2012). These traits are related to lower transpiration rate under well-watered conditions which may result from lower leaf area, lower canopy conductance, and high leaf abscisic acid (Kholova et al. 2010; Ratnakumar and Vadez 2011; Zaman-Allah et al. 2011). Some of the traits which have direct or indirect manifestations under these stresses are as follows, and more detailed discussion has been given in Chaps. 2 and 3.

7.2.1 Morphophysiological Traits

There are several traits which help to make appropriate use of water when it is available and ensure adequate availability to plants during the sensitive phase of floral development and grain growth. In legumes, early plant vigor, fast ground cover and large seed size besides high root biomass, long and deep root system, high leaf water potential, and small leaflets are some of the attributes showing significant association with drought tolerance, whereas high harvest index, large number of pods per unit area, and high seed mass along with early maturity are associated with drought escape (Passioura 1982). In lentil, root length has shown positive correlation with early vigor and SPAD value and therefore can be used as selection criteria for identifying drought-tolerant genotypes (Sarker et al. 2005; Kumar et al. 2012a). Thus genotypes with rapid ground cover, early phenology, a prolonged reproductive phase leading to increased dry-matter production, more pods, high harvest index, efficient water use, and large seeds are targeted in breeding lentil varieties to adapt to drought stress.

In cereals, morphological traits, such as root architecture (Hammer et al. 2009; Lopes and Reynolds 2010; Trachsel et al. 2011) and reduction in unproductive tillers (Mitchell et al. 2013) and physiological ones, such as the translocation of pre-anthesis assimilate to the grain (Bidinger et al. 1977; Blum 1998), are important for breeding drought-tolerant genotypes. A number of other traits including fast seedling establishment (long coleoptiles), rapid ground cover, leaf architecture, root vigor, transpiration efficiency, remobilization to the grain of stem carbohydrates, glaucousness to deflect heat, leaf rolling, and buffering against reproductive failure have been used for improving yield in wheat under drought conditions (Reynolds and Tuberosa 2008; Salekdeh et al. 2009). Stay-green trait and maintaining cool canopies during grain filling also look promising (Jordan et al. 2012; Lopes and Reynolds 2012). Vigorous growth of seedlings is strongly beneficial in cereals as well as legume crops for avoiding the terminal heat and drought (Watt et al. 2005; Kumar et al. 2012a). Deep roots in rice are evidently beneficial (Henry et al. 2011), and in maize better buffering of floret fertility and early grain filling against water stress are likely traits for drought tolerance (Campos et al. 2006). Some of these traits are constitutive and can be selected for in well-watered plants (Richards et al. 2010). In maize, change in color of leaf from green to green-gray and rolling of the lower leaves are important for phenotyping the drought tolerance. Drought-affected ears typically have fewer kernels that will be poorly filled if drought extends throughout grain filling (Edmeades et al. 2000).

Under water and heat stress situation, physiological changes are manifested due to changes in relative water content, water-use efficiency, harvest index, total dry matter, crop duration, transpiration efficiency, etc. Water productivity can be defined at different levels (Condon et al. 2002). At plot level, it can be defined as water-use efficiency (WUE), i.e., total biomass divided by evapotranspiration. At plant level, it is defined as transpiration efficiency (TE), i.e., biomass divided by water transpired, whereas at the leaf level, it is defined as the intrinsic WUE, i.e., the ratio of instantaneous CO_2 assimilation to transpiration. These definitions illustrate that water productivity can be approached from different perspectives, broadly in terms of agronomic and genetic aspects. Regulation of cellular turgor pressure and hydration through osmotic adjustment have shown to increase yield potential

under water-deficit environments. Therefore, analyzing the responses of the physiological determinants of yield to water and heat stresses can be very useful in breeding for high yield and stability in stress-prone environments. Cooler canopy temperature is strongly associated with yield, deeper root system, and stomatal conductance under both drought and heat stress environments (Cossani and Reynolds 2012). Therefore, selection for canopy temperature coupled with early vigor and delayed senescence to improve light interception as well as membrane thermostability and photoprotective pigments and wax to improve radiation use efficiency are desired for heat tolerance screening. However, physiological traits, in general, have seldom been used successfully as selection criteria in breeding programs because of the lack of a simple repeatable large-scale screening technique.

7.2.2 Floral Fertility Traits

Floral fertility can be markedly reduced by water and heat stresses. These stresses can affect the viability of both pollen and ovules (Saini and Westgate 1999; Boyer and McLaughlin 2007; Parish et al. 2012) and may lead to the mistiming of anthesis and silking in maize (Campos et al. 2006). The resulting poor seed set leads to low yields despite vegetative growth being normal. For example, in maize abortion of ovules during water stress depends on the duration of the stress, carbohydrate metabolism, and the induction of senescence genes. Therefore, good survival of pollen among genotypes has been used for drought and heat tolerance in crop plants.

7.2.3 Metabolic Pathways

Drought and heat stresses also affect many metabolic pathways and structures. One commonly observed response of the plant to water stress is the accumulation of metabolically compatible solutes such as proline, glycine betaine, pinitol, carnitine, mannitol, sorbitol, polyols, trehalose, sucrose, oligosaccharides, and fructans in large quantities. Accumulation of these compounds results in decreased water potential, thus facilitating water movement in the cell and helps in maintaining the turgor, which might contribute to sustaining physiological processes such as stomatal opening, photosynthesis, and expansion of growth. However, field studies examining the association between osmolyte accumulation and crop yield have tended to show no consistent benefit under severe water deficit. At cellular level, heat stress leads to membrane damage, denaturation of proteins, inactivation of enzymes in mitochondria and chloroplasts, impaired carbohydrate and protein synthesis, degradation of proteins, synthesis of new proteins, and impaired carbon metabolism (Hasanuzzaman et al. 2013). Heat stress affects the production of sucrose and impairs its transport to influence the developing reproductive organs (Li et al. 2012; Kaushal et al. 2013). High temperature and drought stress can cause denaturation and dysfunction of many proteins such as heat shock proteins and molecular chaperones, as well as late embryogenesis abundant (LEA) protein families, which are involved in plant abiotic stress tolerance.

There is increasing evidence that tolerances to drought and heat are under independent genetic control and can be treated as two distinct traits. Understanding of key genetic controls of root morphology, stay-green trait, and key processes affected by stresses is relatively incomplete. It is generally agreed that drought tolerance from a breeding viewpoint is a complex trait that shows a high level of genotype-environment interaction (Cooper et al. 2006) though from the physiological viewpoint, it can be dissected into several clear processes (Blum 2011). Heat tolerance appears to be less complex, but there is little published evidence to date confirming this assertion (Blum 2011).

7.3 Methods for Phenotyping Drought and Heat Tolerance

The efficiency of a screening technique depends on its ability to reproduce the most probable

conditions of the stress in the target environment (Wery et al. 1994). It requires characterization of the most probable stress in its actual position in the plant cycle and its reproduction in conditions where screening of a large number of genotypes can be made. These two steps are essential for representativeness and reproducibility of screening technique. For example, the terminal drought and heat stress frequently occur at reproductive stage. Therefore, germplasm must be planted at appropriate time in the target location so as to coincide the stress with critical crop growth stages. In addition, the identification of plant traits required for a target environment before screening test is a must. Various approaches have been or are being developed to breed crops for water and heat stress environments with the help of conventional and novel phenotyping methods.

7.3.1 Field Screening

To develop drought- and heat-tolerant germplasm, experiments are conducted either at different dates of planting at a single location or at many locations that cover wide range of water and heat gradients. At ICARDA, the locations for drought and heat screening include Tel Hadya (with a long term average rainfall of 350 mm) and Breda (250 mm rainfall) in Syria and Kfardan (300 mm rainfall) and Terbol (550 mm rainfall) in the Bekaa Valley of Lebanon. Sowing of heat screening trials is delayed to late March in Tel Hadya to allow the flowering and pod development stages to be exposed to high temperature (>35 °C) without any water stress as the crop is irrigated on alternate days or screening of germplasm at the hot spot in Sudan where daily maximum temperature always remains above 35 °C during the reproductive stage. Another strategy adopted for developing drought- and heat-tolerant genotypes is to test elite lines across an artificially created water and heat gradients in fields with rainout shelters. The test entries are planted with repetitive checks (local, susceptible, and tolerant) under well-watered and water-deficit conditions and scored for tolerance using scale 1 (highly tolerant) to 9 (highly susceptible). Evaluation of lentil lines under wooden boxes in plastic house has also been practiced for identification of tolerant genotypes for heat and drought.

Recently, lysimeter facilities that provide soil volumes equivalent to field conditions are established with rainout shelters to study variation in water-use pattern and root depth/density and their relationship to yield under fully irrigated and terminal drought stress (Vadez et al. 2008). Lysimeters which consist of long and large PVC tubes filled with natural soils mimic a real soil profile from the standpoint of volume of soil available and aerial space available for plants. Lysimetric method is used for a yield-based evaluation of genotypes for drought tolerance as this approach allows the monitoring of plant water use and biomass accumulation (both vegetative and grain) from very early plant stages until maturity, and it allows extremely robust TE assessments to be conducted with very low experimental error (Ratnakumar et al. 2009).

7.3.2 Glasshouse Experiments

For drought screening, experiments are conducted in glasshouse under well-watered and stress treatments. Plants are grown in plastic containers/pots in a regular greenhouse and watering is performed manually to allow optimal germination and seedling establishment. Subsequently, the pots are transferred to the "smart house" where each pot is placed onto a cart on a conveyor belt. Every second day, pots are weighed and watered automatically to 22 % gravimetric water content for the well-watered treatment and 15 % for the stress treatment. Based on the experience, we can adjust the drought stress in subsequent experiment. The experiments are carried out under natural lighting with the temperature in the greenhouse kept at a range between 15 °C (night) and 22 °C (day). With the onset of the stress treatment, plant images are captured using a LemnaTec 3D Scanalyzer (LemnaTec, GmbH, Wuerselen, Germany). Every day, three RGB pictures

(2,056 × 2,454 pixels) are taken with a 90° horizontal rotation. Images of other traits like caliper length, height, color and shoot area top view, and convex hull area can also be calculated to estimate the compactness of plant. At the end of the experiment, above-ground biomass, number of branches, and plant height are also determined. Fresh biomass is weighed and subsequently oven dried to constant weight to determine dry weight to calculate water-use efficiency and specific plant weight. In addition, simple stress indices (SSI) are calculated as follows: SSI = Ts/Tc, where Ts and Tc are the average trait performances of a genotype under stress and control conditions, respectively.

7.4 Tools and Technologies for Phenotyping Drought and Heat Tolerance

7.4.1 Tools for Measuring Root Traits

Root systems are inherently difficult to study due to their underground environment, the complexity of dynamic interactions between roots and its environment, and the diversity of their functions. This trait has been discussed as underground trait in Chap. 8. Harper et al. (1991) classified available methods to study roots into two groups: destructive and nondestructive methods. The main criterion determining the selection of a method to study roots depends on whether the focus is on changes in root traits over time or space. For the study of changes of root traits over time, nondestructive methods are recommended. A cubic section of soil that contains roots (i.e., monolith) is dug out from the soil or obtained from a container in which the plant has been grown. Afterward, the monolith is washed to remove soil and separate roots. Although the root system may be damaged during sampling, a representative characterization of its morphology can be obtained. Predictive techniques provide insight about root systems by extrapolating root information from soil cores and root crowns of field-grown plants (Trachsel et al. 2011) or from plants grown in controlled growth systems including hydroponic, pouch, pots, and plate systems. In situ methods involving rhizotron, magnetic resonance, and compound tomography techniques have also been developed to facilitate nondestructive spatial and temporal investigations (Taylor et al. 1990; Gregory et al. 2003; Tracy et al. 2010); however, the current scale, resolution, throughput, and cost-efficiency of these techniques limit their utility (Clark et al. 2011). Additionally, simulation and modeling studies that integrate rhizosphere and growth data help form links between predictive techniques and field studies, allowing researchers to strategically predict, evaluate, and target beneficial root traits or genotypes for specific growth environments.

As a complimentary tool to other predictive techniques, gellan gum growth systems with superior optical clarity (Clark et al. 2011) have been introduced to facilitate noninvasive two-dimensional (Iyer-Pascuzzi et al. 2010) and three-dimensional (Fang et al. 2009) imaging and temporal studies of plant root systems while also allowing reproducible control of the rhizosphere. In the two-dimensional (light, X-ray, neutron radiography) and three-dimensional (X- or NMR-based tomography) rhizotrons, plants are grown in a flat container with side walls made of a transparent material such as glass. Optical scanners can also be used to process samples obtained by soil coring or by burying them in the soil to study roots in a similar way as with 2D rhizotrons. Minirhizotrons are small-diameter transparent tubes inserted into the soil for root observation. X-ray, γ-ray, thermal neutron, and magnetic resonance tomography (computed tomography methods) allow roots growing in the soil media to be imaged noninvasively (Johnson et al. 2001; Tracy et al. 2010). The total root length per segment can be determined using the WinRHIZO Tron MF software (Regent Instruments Inc., Canada). Direct selection for roots is not yet feasible at a breeding scale due to high cost and low throughput of current methodologies. Recently, ground penetrating radar (GPR), a remote-sensing technology, has been successfully used in evaluation of coarse tree root biomass (Butnor et al. 2003).

Incorporating GPR (SIR-20 GPR) into current crop phenotyping methodologies could potentially provide a long awaited solution to high-throughput phenotyping for roots under realistic field conditions.

7.4.2 Spectral Reflectance

Phenotyping tools based on spectral reflectance provide fast, nondestructive measurements of green biomass, canopy chlorophyll content, leaf and canopy senescence (or stay green), and plant water status (see Chaps. 4 and 5). Portable leaf chlorophyll meters such as the SPAD meters measure optical density differences between two wavelengths within the red and the near-infrared regions (650 and 940 nm) and have been used as an indirect measurement of leaf N and chlorophyll content. The SPAD chlorophyll meter readings (SCMR) are recorded at equivalent cumulative thermal times. After recording the SCMR, the leaves are processed by soaking in water to bring to full turgor for specific leaf area (SLA) measurement. The leaf area can be measured with a leaf area meter (LI-COR Area Meter). However, SPAD measurements are taken on individual leaves, with a very small measurement area (2 mm $\times$ 3 mm). At the canopy level, the normalized differential vegetation index (NDVI) of the light reflected by the canopy has been used to quantitatively assess plant growth and senescence (Aparicio et al. 2000; Marti et al. 2007). Fast measurements of NDVI can be performed using spectroradiometers provided with active sensors. This is the case of the GreenSeeker (from N-Technologies) which is a relatively low-cost spectroradiometer designed to allow fast measurements of NDVI.

7.4.3 Imaging Technologies

With noninvasive imaging of plant growth, it is now possible to quantify growth-related parameters, detect stress symptoms and their timing, as well as estimate the recovery of growth after the stress (see Chaps. 4 and 5 for details). Several types of plant images can be taken, e.g., with infrared, near-infrared, fluorescent, and visible light. Scanning with infrared light gives information on plant or leaf temperature, while near-infrared imaging sheds light on the plant water content and fluorescent pictures enable conclusions on plant health status. High-resolution color picture (RGB picture), taken from the top and two side views, is used to determine the projected shoot area of the plant. The projected shoot area serves as a measure for biomass. Hence, from RGB images taken at several time points, growth curves as well as growth rates can be calculated. Digital imagery can be used for estimating leaf nitrogen content, early biomass, and response to water-limited conditions. Its low cost and the small amount of technical experience required make it very useful for phenotyping drought tolerance and other complex traits (Rorie et al. 2011).

Measurement of chlorophyll fluorescence is another simple and reliable technique for screening germplasm for drought traits and has long been used to examine various photosynthetic parameters in leaves (Baker 2008). The chlorophyll fluorescence can be measured by the dark-adapted test of the modulated chlorophyll fluorometer OS1-FL (Opti-Sciences, Tyngsboro, MA, USA). With this system, chlorophyll fluorescence is excited by a 660 nm solid-state light source with filters blocking radiation longer than 690 nm. The average intensity of this modulated light is adjusted from 0 to 1 mE. Detection is in the 700–750 nm range using a PIN silicon photodiode with appropriate filtering to remove extraneous light. The clamps of the instrument are installed on the leaves to keep them in the dark and to stop the light reaction of photosynthesis for 45 min. After this, the clamps are attached to the optic fiber of the device and the valves of the clamps are opened. After starting the device, the 695 nm modulated light is radiated through the optic fiber toward the leaf. Subsequently, the chlorophyll fluorescence expressed as Fv/Fm ratio is recorded. The leaves tested for chlorophyll fluorescence are also used for measurement of chlorophyll content.

Infrared thermography (IRT) provides a powerful imaging tool for rapid, noninvasively, and remotely measuring leaf temperature as a surrogate for stomatal conductance. Plant temperature also allows the calculation of the crop water stress index (CSWI). Water stress and CSWI have been linked to soil water availability, leaf water potential, stomatal conductance, and yield (Romano et al. 2011). The use of thermography to determine stomatal conductance has been optimized through the development of standard protocols which take into account the surrounding environment and even the distribution of stomata between the two leaf surfaces (Guilioni et al. 2008). Stomatal conductance and leaf temperature of fully expanded leaves can be measured using a portable leaf porometer (model SC1; Decagon Devices, Pullman, WA, USA). Leaf temperature can be recorded with infrared sensor (Oakton Instruments, Vernon Hills, IL, USA). Thermography has become a standard technique to determine stomatal conductance in both glasshouse (Grant et al. 2006) and field environments (Grant et al. 2007).

A novel imaging system that incorporates measurements of chlorophyll fluorescence and thermal imaging under controlled gaseous conditions is developed which offers a noninvasive, high-throughput, high-resolution tool to screen intrinsic water-use efficiency (McAusland et al. 2013). This new imaging system generates images of assimilation rate (*A*), stomatal conductance (g_s), and intrinsic water-use efficiency (WUE_i) from whole plants or leaves under controlled environmental conditions. A major advantage of the combined imaging approaches is the ability for multiple samples to be measured at any one time and the fact that spatial heterogeneity within plants and leaves can be readily identified.

7.4.4 Stable Isotopes

Traditional methods of determining WUE are unsuitable for rapid screening for several reasons. These agronomic techniques are not only destructive but also rely on an integrated measurement of biomass/yield at the end of the growing season relative to the amount of water used over the growing period. Carbon isotope discrimination has been successfully used to identify crop cultivars with greater WUE (Farquhar et al. 1982; Condon et al. 2004); however, this technique also relies on an integrated measure of WUE over a period of plant growth. Additionally, the technique does not provide an indication of whether differences in WUE are driven by CO_2 assimilation (A) or water loss, although the incorporation of oxygen isotope measurements can provide an indication of rates of evaporation from the leaf surface (Farquhar et al. 1998; Barbour 2007). Leaf-level gas exchange measurements of the rate of CO_2 assimilation relative to transpiration provide an immediate and nondestructive measure of instantaneous WUE (Penman and Schofield 1951) or intrinsic water-use efficiency (WUEi), when stomatal conductance (g_s) is used instead of transpiration as a measure of water loss. Although this approach is flexible in terms of the timescale of when measurements can be made, an infrared gas analyzer (IRGA) can only take singular measurement on one plant or leaf at one point of time. The natural variation in plant N isotope composition (Δ15N) is potentially useful for genotypic screening under drought (Cernusak et al. 2009; Tcherkez 2011). For a large-scale phenotyping, the above isotopic signatures may be estimated in a fast and low-cost manner by near-infrared reflectance spectroscopy (NIRS) (Ferrio et al. 2007; Cabrera-Bosquet et al. 2011). This tool has also been reviewed in Chap. 3.

7.4.5 Integrated Automatic Phenotyping Platform

Great efforts have been made in recently established plant phenotyping facilities for developing rapid phenotyping techniques. These facilities aim to explore traits postulated to improve resistance to a given stress or to discover new ones. First these traits are often explored initially in controlled laboratory environments and may not be well connected to the way plants

behave season-long in the field (Passioura 2010). In fully automated greenhouses, plants can be delivered via conveyor belts to watering, weighing, and imaging stations. In these high-throughput phenotyping (HTP) facilities, several hundred individual plants can be imaged per day in a fully automated manner. HTP facilities of this type are currently in use in various research institutes (The Plant Accelerator, Adelaide, Australia; CropDesign, Gent, Belgium; IPK Gatersleben, Germany; PhenoArch, Montpellier, France) and separately discussed in Chap. 19. Such phenotyping facilities are ideal to combine controlled irrigation and phenotyping protocols. Canopy temperature is an ideal HTP field screen for heat tolerance as it allows rapid estimation of difficult phenotype traits such as transpirational flux and root depth (Lopes and Reynolds 2010). In addition, infrared and other remote spectral-sensing platforms are being continually refined, allowing canopy temperature, pigment composition, hydration status, and ground cover to be measured on a breeding scale (Cossani and Reynolds 2012). More direct procedures for HTP of root structure are also under investigation, including ground penetrating radar, X-ray, γ-ray, thermal, neutron, and magnetic resonance tomography (Tracy et al. 2010). Integrated use of spectral reflectance spectroscopy and other remote-sensing HTP methods with simulation models, incorporating field, and environmental data will make extensive screening of genetic resources more feasible for drought and heat stresses.

7.5 Conclusions

To date, breeding for drought and heat tolerance has been based principally on empirical selection for yield per se. However, this approach is far from being optimal since yield is characterized by low heritability and high genotype-environment interaction. Therefore, rapid progress directly depends on our ability to precisely target key traits and to identify and locate genes/QTLs controlling them. Physiological traits that contribute to improved productivity under drought and heat conditions need to be the focus of the future research. Many new promising tools for evaluating physiological traits are now available. Current physiological tools used for high-throughput phenotyping with focus on field-based methodologies are based on remote-sensing techniques designed to assess plant performance in a fast and nondestructive manner and holds great promise.

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Phenotyping for Root Traits

8

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Abstract

Root system architecture determines crop capacity to acquire water and nutrients in the dynamic and variable soil environment. Increasing attention is paid to searching for optimal root traits to improve resource uptake efficiency and adaptation to heterogeneous soil conditions. This chapter summarises genetic variability and plasticity in root traits relevant to increased efficiency of soil resource acquisition. Approaches available for high-throughput phenotyping of root architecture traits at both laboratory and field scales are critically assessed. The advent of several novel imaging technologies such as X-ray computed tomography coupled with image-analysing software packages offers a great opportunity to non-invasively assess root architecture and its interactions with soil environments. The use of three-dimensional structure–function simulation root models is complementary to phenotyping methods, providing assistance in the crop breeding programmes. We also discuss applications and limitations of these novel visualisation technologies in characterising root growth and the root–soil interactions.

Keywords

Root traits • Variability • Plasticity • Hydroponics

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8.1 Introduction

Root system is a fundamental component of plants. Apart from mechanical support to the above-ground parts, root system is also the major organ for foraging and acquiring nutrients and water from the surrounding soil. Root system is a complex three-dimensional (3D) structure exhibiting a specific spatial and temporal configuration of root types. Root system architecture (RSA) refers to in situ spatial distribution of the root system within the rooting volume (Hinsinger et al. 2011; Lynch 1995, 2007; Manschadi and Manske 2013). RSA plays a vital role in the exploration of soil zones and acquisition of soil water and nutrients (Gregory et al. 2009; Hammond et al. 2009; Lynch and Brown 2012). It is plastic and dynamic, allowing plants to respond to their environments in order to optimise acquisition of important soil resources (Zhu et al. 2011).

Studies of RSA are concerned typically with an entire root system of an individual plant, rather than just fine details of the root structure (Lynch 1995). The root architectural traits include three general categories: topological properties (describing the pattern of root branching), geometric properties (the presence of roots in a spatial framework, such as the growth angle of root axes) and physiological properties (such as root growth rate, root exudation and root water and nutrient use efficiency) (Chen et al. 2011a; Gregory 2008; Manschadi et al. 2008). Crop adaptation to suboptimal soil conditions is dependent on RSA, and thus crop survival and fitness are determined by the RSA (Eshel and Beeckman 2013; Fitter et al. 2002; Lynch 1995). However, as 'the hidden half' of a plant, root system is often underappreciated largely due to the inherent difficulty of accessing it for studies (Eshel and Beeckman 2013; Smith and De Smet 2012).

Exploiting genetic diversity in root traits associated with acquisition of scarce soil resources and adaptation to edaphic stresses can significantly enhance resource use efficiency in crop plants and thus lead to improved productivity. It might be advantageous for a plant to have the root system with architectural traits specifically adapted to the prevailing soil conditions (Trachsel et al. 2013). The identification of relevant root traits offers the potential to increase the grain yield of not only crops growing soil resources but also crops growing with optimal water and nutrient supply by revealing physiological traits associated with the partitioning of dry matter.

The identification of optimal root traits under stress environments depends on targeting the probable stresses that the crop may face during the growing season. Targeted development of crop genotypes with increased efficiency of nutrient capture (Rengel 2005; Wu et al. 2005) and water use (Kamoshita et al. 2000; Liu et al. 2007; Manschadi et al. 2006; Ober et al. 2005; Rengel 2013) relies on a better understanding of root structure and functions and the exploration of optimal root traits for specific growth environments (Wang and Smith 2004). It has been demonstrated that modification of root architectural traits could contribute to improved grain yield, drought tolerance and resistance to nutrient deficiencies (Beebe et al. 2006; Steele et al. 2006; Tuberosa et al. 2002a).

Quantitative genetic studies require efficient phenotyping protocols (Trachsel et al. 2013). However, the inability to efficiently and accurately phenotype large mapping populations has been a key impediment to wide-scale use of root-related genetic information in breeding (Chen et al. 2011a; De Dorlodot et al. 2007). Hence, accurate phenotyping of root-related traits is one of the most important practices for translating into breeding programmes the recent physiological and genetic advances in understanding the role of root systems in improving crop yield and productivity in dry environments. However, phenotyping of root traits requires multidisciplinary analysis because the root structure and function and their responses to heterogeneous soil environments are dynamic and complex (Doussan et al. 2003; Hodge 2004; Pierret et al. 2006; Valizadeh et al. 2003). Hence, phenotyping for optimal root traits is often conducted under controlled environmental conditions, whereas systematic phenotyping for root traits in the field remains challenging (Fiorani and Schurr 2013; Trachsel et al. 2011).

In the recent decade, some novel non-invasive and high-throughput phenotyping technologies have been developed for fast, accurate and robust analyses of root structure and function. In addition, current simulation computer models offer a promise in characterising intrinsic genetic properties and phenotypic plasticity of root traits in large-scale phenotyping required in breeding for improved productivity (Dunbabin et al. 2013; Struik and Yin 2007).

This chapter discusses genetic variability and plasticity in root traits relevant to increased soil resource use efficiency and better adaptation to specific soil environments, followed by an overview of recent developments in high-throughput phenotyping methods. This review also highlights applications and limitations of some novel visualisation technologies and modelling simulations in characterising root growth and the root–soil interactions.

8.2 Root Trait Variability

Genotypic variability and phenotypic plasticity are the two general types of variability in root architectural traits. These are derived from two different developmental pathways, namely, genetically determined intrinsic pathway (governing the basic architecture and the limits of plasticity) and environmentally triggered responsive pathway (Malamy 2005). These two pathways combine in intricate ways to create a highly complex 3D root structure influenced by genetics as well as the availability of resources in the heterogeneous soil environment (Baddeley et al. 2007).

8.2.1 Genotypic Variability in Root Traits

Variation in root architectural traits critically influences the capacity and efficiency of a plant in foraging and taking up water and nutrients from soil. For example, changes in the root system architecture in response to low phosphorus (P) availability may enhance P uptake (Nielsen et al. 2001). Shallow rooting is beneficial for P uptake because in many soils, most of the available P is retained in the topsoil layers. In soybean (*Glycine max*) genotypes, increased yield potential was linked to increased capacity to take up water from deep (1.1 m) soil horizons in the field (Ober et al. 2005); similar connection was established for upland rice (*Oryza sativa*) in Laguna, Philippines (Kamoshita et al. 2000; Kondo et al. 1999), and wheat (*Triticum aestivum*) in Western and Southern Australia (Wong and Asseng 2006; Manschadi et al. 2010). Understanding the role of and manipulating root length branching at depth and seminal root angles have been flagged as key factors likely to underpin further increases in wheat yield (Manschadi et al. 2010).

Our recent studies examined genotypic variability in a large germplasm collection of narrow-leafed lupin. Wild genotypes with contrasting root architecture differed in root growth, root distribution in the profile and P acquisition in response to localised P supply (Chen et al. 2013a). Selected genotypes differed in root length density (root length in a unit soil volume).

Specifically selecting for improved root traits, such as root proliferation at depth, may contribute to increased productivity in crops, especially in dry soil conditions, and in soils with high strength because of natural settling or formation of a shallow hardpan due to vehicle movement (Hall et al. 2010). This is particularly important because attempts to increase root density at depth using agronomic approaches (e.g. deep fertiliser placement and ripping) have been largely unsuccessful (e.g. Baddeley et al. 2007).

There is little knowledge on genotypic variability in root function related to the architecture. For example, wild genotypes of narrow-leafed lupin (*Lupinus angustifolius*) exhibited genetic variation in exudation of organic acid anions into the rhizosphere (Chen et al. 2013b). We observed that, at optimal P, the large-rooted genotype exuded citrate, acetate and malate, whereas the other two genotypes with smaller root systems only released citrate in significant amounts. The significance of these findings in a relationship between root architecture and functions is yet to be assessed.

8.2.2 Phenotypic Plasticity in Root Traits

Several mechanisms may be involved in regulating root architecture alteration in response to heterogeneous nutrient availability. Examples of such mechanisms are root proliferation in localised nutrient-rich patches, changes in rooting depth or the relative length of different root diameter classes (e.g. Dunbabin et al. 2001a; Ho et al. 2005; Paterson et al. 2006; Smith and De Smit 2012). Plant changes in root morphology in response to changes in P availability are an essential strategy for efficient P acquisition (Chen et al. 2013b; Lambers et al. 2011).

Crop genotypes often exhibit variable capacity to alter the growth, number, size and distribution of the root systems in order to optimise resource capture when exposed to heterogeneous environmental conditions. Phenotypic plasticity was evidenced in root length density in the lupin genotypes in response to varying P supplies (Fig. 8.1; see also Chen et al. 2013a). For example, in comparison with the nil-P treatment, the large-rooted genotype produced significantly more branches in the top 30 cm of the soil profile when P was placed in a band 10 cm below the soil surface. Such developmental plasticity is based on the capacity of plant cells either to remain undifferentiated until the root-growth response is initiated or to dedifferentiate into cells that can grow into new roots.

In terms of root mass allocation, many *Lupinus* species with a high capacity to acquire P exhibit low root-growth plasticity at low P supply (Pearse et al. 2006). On the other hand, some *Lupinus* species increase root–shoot biomass ratio during water stress (Carvalho et al. 2004).

8.3 Phenotyping Systems

Crop breeders and researchers are showing increased interest in phenotyping for root architecture traits as part of their breeding programmes. High-throughput phenotyping for root architecture traits requires fast, reliable and accurate root observations and measurements. Recent progress in the development of root-related methodologies, from traditional excavation to modern non-destructive imaging technologies including X-ray computed tomography (CT), has significantly enhanced our capacity to visualise, quantify and conceptualise root architecture and its relationship to (1) crop adaptation to variable growth environments and (2) plant productivity (Gregory et al. 2009; Iyer-Pascuzzi et al. 2010; Lynch 1995). It is still a challenge to elucidate the genetic and developmental basis of the root system architecture, and a combination of laboratory- and field-based approaches should be considered (Clark et al. 2011). In this section, we overview current phenotyping systems employed in the controlled environments and the field (Table 8.1) and discuss their applications and limitations.

8.3.1 Controlled Environments

A number of phenotyping approaches are available for (1) destructive and (2) non-destructive sampling of root systems. Destructive sampling by excavating whole root systems from soil-filled containers (e.g. pot, columns, boxes, tubes and chambers) is used commonly in root studies in the controlled environments (Table 8.1). In the recent decades, non-destructive approaches have been developed for high-throughput phenotyping of root architecture traits with the support of advanced optical recording techniques. These include (1) soil-filled rhizotrons with clear panels for root observations and (2) soil-free approaches in artificial media, such as hydroponics, aeroponics and the gel chamber or agar-plate systems.

8.3.1.1 Soil-Filled Pots

The soil-filled pots provide environments for crop growth that may to some extent simulate those in the field. Various root traits can be measured, including total root length, root mass and root density per soil volume; also, roots at various depths in the soil profile can be measured

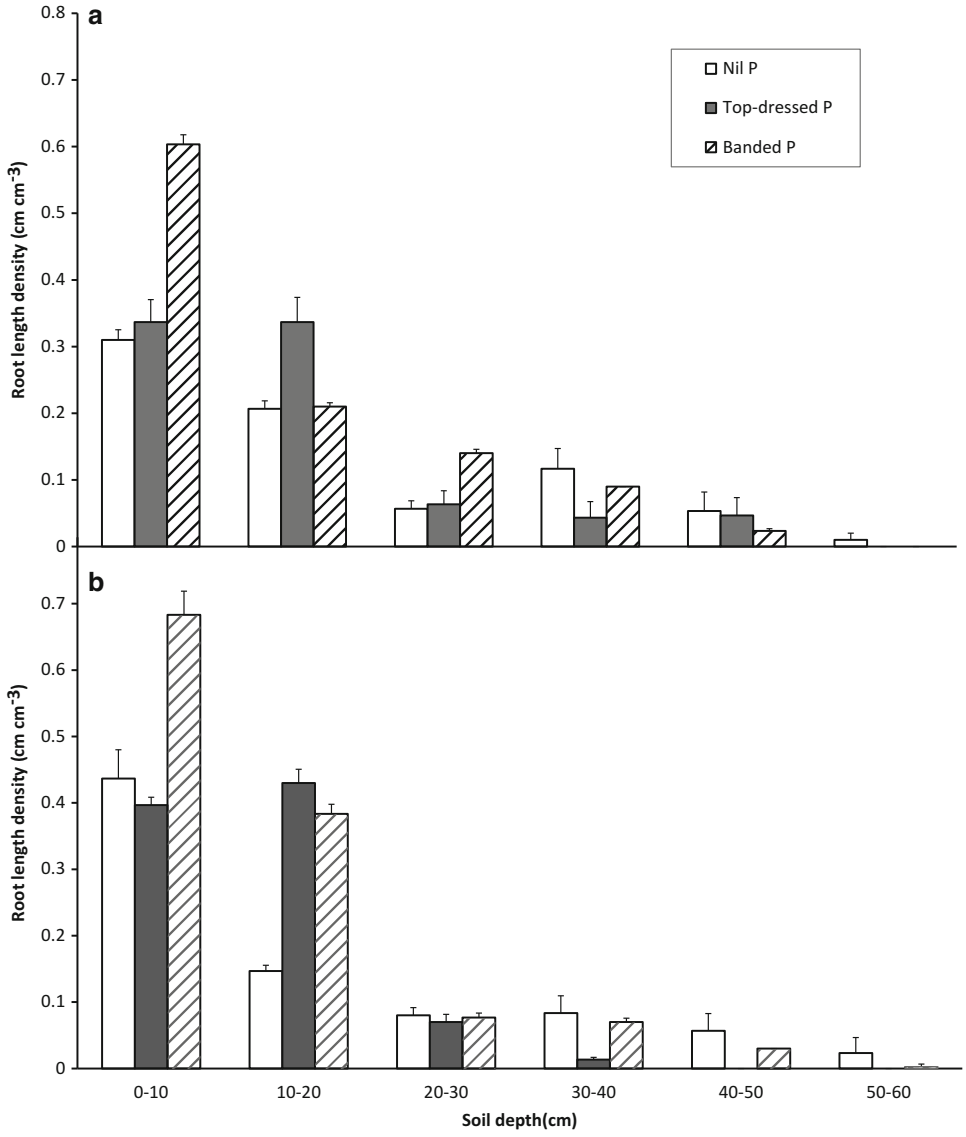

Fig. 8.1 Variability in root length density (cm cm^{-3}) in the soil profiles of two wild genotypes (**a** #071; **b** #085) of *Lupinus angustifolius* grown in a glasshouse for 42 days under three P-application treatments: nil P (no fertiliser P application), top-dressed P (fertiliser P applied on the soil surface) and banded P (fertiliser P placed in a narrow band 10 cm below the soil surface). Data are means + s.e. (*n*?=?3) (Modified from Chen et al. 2013a)

(Araki and Iijima 1998). However, this method requires destructive sampling of roots and involves the process of root washing out of soil (Hund et al. 2009b). Hargreaves et al. (2009) placed a starch-based polymer net sac in the centre of the soil-filled plastic pot and observed genotypic variation in root numbers, length, mass and root angles (i.e. vertical spread) of lateral roots in five barley genotypes. The soil sac method could be improved to allow more systematic construction and data collection. However, this method, similar to the standard pot method, is destructive. Despite all due care, roots could be broken inside the netting so their coordinates could not be taken, jeopardising measurements of root spread.

Table 8.1 Summary of methods for phenotyping root traits

Growth medium	Phenotyping system/ method	Crop species	Capacity	Traits observed	Merits	Limitations	Example reports
Agarose gel or gellan gum	Agar-solidified nutrient solution in Petri dishes; transparent gel or gellan gum on transparent plastic plates or in ungraduated borosilicate cylinders or glass cylinders/ chambers	Barley (*Hordeum vulgare*, *H. spontaneum*), rice (*Oryza sativa*), canola (*Brassica napus*), *Arabidopsis* (*Arabidopsis thaliana*), timothy (*Phleum pratense*), tomato (*Lycopersicon lycopersicum*), lettuce (*Lactuca sativa*), alyssum (*Aurinia saxatilis*)	Up to a large number of genotypes (hundreds)	Root length, number, elongation rate; vertical angular spread of roots; root hairs, diameter, surface area, volume, distribution; relative elongation rates between the elongation zone and the meristem; some 27 static/dynamic and global/local root traits calculated with the RootReader3D software based on 3D images	The gel/gellan-gum system provides easy, fast, non-destructive and repeated measurements of 2D (via a flatbed scanner) or 3D (using RootReader3D software) root system. It can be applied to hundreds of plants, to create a uniform and reproducible environment for root growth, easy handling with minimal risk of accidental damage to roots. The 2D analysis is simple to record and analyse and would be simple to model. The use of gellan-gum systems has an enormous potential for high-throughput root phenotyping and novel trait discovery	Anaerobic conditions in agar may alter plant growth. The 2D system with artificial environments may not reliably represent the 3D soil environment. The size of the chamber may restrict its use to studies of young seedlings only. Efforts to expand the 2D images into 3D structure remain constrained by low throughput (>1 h to image a single root system), small scanning volumes and limited quantification capabilities	Bengough et al. (2004), Clark et al. (2011), Fang et al. (2009), Hargreaves et al. (2009), Iyer-Pascuzzi et al. (2010), Shiet al. (2013), van der Weele et al. (2003)
Nutrient solution	Hydroponics (roots in solution); semi-hydroponics (roots in air and moisture provided by automatic irrigation system)	Maize (*Zea mays*) narrow-leafed lupin (*Lupinus angustifolius*)	Up to a large number of genotypes (hundreds)	Root length, number, elongation rate, hairs, diameter, surface area, volume, mass	High throughput; can reproduce 2D or 3D root systems; simple and fast measurements of dynamic root growth. The semi-hydroponic platform permits non-invasive, repeated imaging of	Plants may perform differently in hydroponics and soil. Hydroponic method does not reflect heterogeneous distribution of nutrients often present in real soils	Chen et al. (2011a, 2012), Sanguineti et al. (2006)

					dynamic root growth and allows phenotyping of relatively large sets of genotypes for a long growth period in a small space		
Germination paper/ blotting paper / growth pouch	The paper pouches are placed upright into plastic containers with nutrient solution	Common bean (*Phaseolus vulgaris*); maize (*Zea mays*)	Small number of genotypes	Root length of basal roots; taproot length per vertical layer; growth angles of basal roots (reflecting root gravitropism); lateral root length and number; other common root traits obtained via WinRhizo software based on 2D root images	Simple, non-destructive, 2D observation of root growth during the early days. Root growth receives lower mechanical impediment than in soil. The technique can be used to analyse kinematics of various root systems using KineRoot image-analysing software	Root growth is forcibly two dimensional. Phenotyping large sets of genotypes beyond early growth stages remains problematic	Basu et al. (2007), Hund et al. (2009a, b), Liao et al. (2004)
Sand, soil or artificial solid substrates (laboratory)	PVC or Perspex cylinders/columns, tubes or boxes or transparent rhizoboxes filled with soil or artificial solid substrate; soil sacs consisting of a plastic pot with a starch-based polymer net sac; root growth assessed via 2D imaging by a camera or flatbed scanner, 3D image by X-ray scanner, or excavation	Barley (*Hordeum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*)	Small number of genotypes	Root number, length (at depth), vertical spread angles, cortical aerenchyma	The soil sacs provide a simple method to compare root growth against the gel chambers and X-ray pots. The X-ray method allowed in situ non-destructive and sequential data collection. An advantage of the X-ray scanner was its facility to track root growth inside the soil throughout the pot, showing clearly that the angles of roots change during growth	The excavation method remains labour intensive and does not allow for high throughput. Artificial systems fail to mimic the complex interaction between the plant and intrinsic abiotic and biotic soil properties. The soil sac method is destructive and data are only obtained where the roots pass through the net boundaries. Roots may be broken inside the netting. It is also	Araki and Iijima (1998), Hargreaves et al. (2009), Hund et al. (2009a), Watt et al. (2006), Zhu et al. (2010)

(continued)

Table 8.1 (continued)

Growth medium	Phenotyping system/ method	Crop species	Capacity	Traits observed	Merits	Limitations	Example reports
						possible that the netting could impede growth as roots may face resistance on reaching the netting, forcing deviation or diameter expansion. The X-ray pots required some patience to set up accurately	
Soil (field)	Excavation/ shovelomics; installation of minirhizotron in soil	Maize (*Zea mays*), common bean (*Phaseolus vulgaris*), narrow-leafed lupin (*Lupinus angustifolius*)	Small to medium number (up to 50) of genotypes	Root cortical aerenchyma, length, number at depth; branching density of brace roots; root angles;	Provide real plant growth environments; shovelomics techniques provide high-throughput assessment of the tested crop species	Application of shovelomics techniques for other crop species needs further examinations. These methods remain labour intensive. Accurate analysis is also compromised due to the loss of some fine and broken roots during the process. Information on root architectural properties	Araki et al. (2000), Chen et al. (2014), Postma and Lynch (2011), Trachsel et al. (2011), Zhu et al. (2010)
Soil (field)	DNA-based quantification method (qRT-PCR typing)	Wheat (*Triticum aestivum*); rice (*Oryza sativa*)	Small to medium number (up to 50) of genotypes	Root DNA density in soil	High throughput	DNA-based techniques only detect and quantify root mass presented in soil samples	Huang et al. (2013), Topp et al. (2013)

8.3.1.2 Rhizotrons

Root observation rhizotrons (rhizoboxes or root chambers) are similar to the soil-filled pot method mentioned above, except that clear acrylic glass panels allow visual monitoring of root growth at the surface of the glass (Manschadi et al. 2006, 2008; Wiese et al. 2005). Variation in root growth and morphology among the tested crop plants can be traced on the outside surface of the acrylic glass using a marker pen – different colours may be used to indicate the presence of roots at successive time intervals, followed by photographing or scanning for root quantification (Fig. 8.2).

Rhizotrons can be designed and constructed to meet specific research needs, such as for deep-rooted crops and long growth periods. For example, Manschadi et al. (2006) used 240-cm-wide, 120-cm-deep root chambers in examining spatial patterns of root length distribution in a drought-tolerant wheat genotype compared with the standard wheat variety at anthesis. Rhizotron systems artificially restrict root growth to two dimensions only. In addition, they suffer from the general disadvantages of pot experiments associated with the disturbed soil structure, altered root-zone temperatures and the limited rooting volume.

8.3.1.3 Agar (Gel, Gellan-Gum) Systems

The agar/gel method, a useful non-soil system, allows quick and easy measurement and visualisation of dynamics of early root growth in seedlings. Root architecture traits can be non-destructively recorded in two dimensions (2D) or 3D using flatbed scanning, digital cameras or X-ray cameras through the transparent substrate. This method has been used for high-throughput phenotyping of root architecture traits in various crop species, such as barley (wild, *Hordeum spontaneum*; domesticated, *H. vulgare*) (Bengough et al. 2004; Hargreaves et al. 2009) and rice (*Oryza sativa*) (Clark et al. 2011; Iyer-Pascuzzi et al. 2010).

The agar/gel method permits detailed characterisation of root traits and root development; for example, the pattern and timing of lateral root initiation were characterised in *Arabidopsis* seedlings (Dubrovsky et al. 2006).

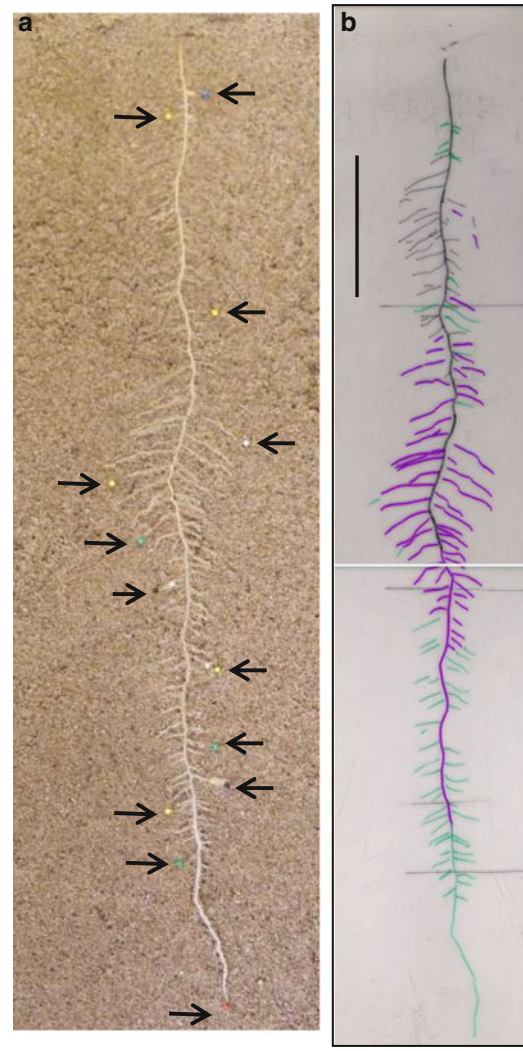

Fig. 8.2 Example of two-dimensional root images of a wild genotype of narrow-leafed lupin (*Lupinus angustifolius*) grown in a soil-filled rhizotron as part of a phenotyping experiment to determine genotypic variability in root growth and temporal–spatial exudation among wild and commercial varieties. (**a**) A rhizotron with the acrylic glass panel removed to expose root system for sampling root exudates around individual root tips using the anion exchange membrane (AEM) indicated by inserted *pins* and *arrows*. (**b**) Root image of the same plant is (**a**) acquired by a flatbed scanner via scanning the acrylic glass with traced root morphology at three consecutive times: 14 (*black*), 20 (*purple*) and 26 (*green*) days after sowing (Bar = 10 cm) (Chen and Rengel 2014)

A new algorithm for computational image analysis of deformable motion at high spatial and temporal resolution was applied to contrasting root growth in *Arabidopsis*, timothy (*Phleum pratense*), tomato (*Lycopersicon lycopersicum*), lettuce (*Lactuca sativa*) and alyssum (*Aurinia saxatilis*) (van der Weele et al. 2003) using the gel system.

By incorporating image-analysing tools, e.g. RootTrace (see Sect. 1.4; Table 8.2), the agar/gel method enables high-throughput, automatic tracing of root growth in *Arabidopsis* seedlings to quantify root length, curvature and stimulus response parameters such as onset of gravitropism (French et al. 2009). The gel plate system offers a moderately rapid screening method for seedlings, the results of which appear to reflect angular root spread in 3D, though care must be taken to avoid contamination problems and use of inappropriate media. The gel plate method is also of particular benefit for non-destructive monitoring of seedling root growth. Hargreaves et al. (2009) compared root growth in gel chambers, soil sacs and X-ray microtomography pots and arrived at similar (albeit not identical) ranking order of angles using the three methods.

Earlier, limitations of the gel chamber system have been outlined (see Futsaether and Oxaal 2002; Hargreaves et al. 2009). These include the chamber size, restricting the method to studies of young seedlings and the fact that it is an artificial environment. Moreover, gel chambers generate 2D data as opposed to the 3D environment encountered by most plants, and anaerobic conditions in agar may alter plant growth (Clark et al. 1999; Hargreaves et al. 2009).

The use of gellan-gum growth systems with superior optical clarity also facilitates non-invasive 2D (Iyer-Pascuzzi et al. 2010) and 3D (Fang et al. 2009) imaging and temporal studies of root systems while allowing reproducible control of the rhizosphere. Topp et al. (2013) employed nutrient-enriched gellan gum to grow rice and demonstrated the capacity of a semiautomated 3D in vivo imaging and digital phenotyping pipeline to interrogate the quantitative genetic basis of the root system. The study phenotyped 25 root traits governing the distribution, shape, extent of exploration and the intrinsic size of root networks at three observation times during the seedling stage. While these recent studies demonstrate the use of gellan-gum systems for potential high-throughput root phenotyping and novel trait discovery in 2D, efforts to expand these investigations into the 3D structure remain constrained by the long scanning times, small scanning volume and limited quantification capability (Clark et al. 2011). Hence, the agar/gel and gellan-gum methods work well for simple root systems, but obscure the more complex 3D root architectures.

8.3.1.4 Hydroponics

Growing plants hydroponically is widely used in seedling studies. The inexpensive, space-saving, high-throughput (semi-) hydroponic system offers the advantage of growing a large number of plants under uniform conditions, two important prerequisites for investigating quantitative traits, particularly those of low heritability. Measuring traits at the seedling stage in hydroponic culture eliminates the challenges of soil contamination and root loss during washing (Chen et al. 2011a).

Seedling traits of 47 commercial maize (*Zea mays*) hybrids were screened using nutrient solution (Sanguineti et al. 2006). Singh et al. (2013) reported a new hydroponic phenotyping technique in examining survivability and drought tolerance of 15-day-old seedlings of 80 genotypes of lentil (*Lens culinaris*).

A novel semi-hydroponic phenotyping platform developed by Chen et al. (2011a) has the potential in studying root response and plasticity in morphological and physiological responses to water and nutrients because the supply of water and nutrients can be adjusted easily. This high-throughput phenotyping system was designed for characterising variability in root architectural traits of narrow-leafed lupin (Chen et al. 2011a, b, 2012). The system is based on a 240-L mobile bin and allows root growth up to 1-m depth, with repeated observations and measurements of 2D root structure without the need for destructive sampling (Fig. 8.3). It is notable that this system

Table 8.2 Selected software packages for root image analyses

Software	Brief descriptions	Root traits	Licence	Automation level	Plant requirement	Image requirements	Source	Website
DART	DART (Data Analysis of Root Tracings) can display root growth sequences over time. It generates structured and flexible datasets of individual root growth parameters and thus enables studying root system architecture	Root length; topological properties; insertion	F	Manual	Seedlings to mature plants	Any	Le Bot et al. (2010)	www.avignon.inra.fr/infos/404
Delta-T-Scan	The software package allows fast and accurate root measurements, including root tip count, with high resolution over large area and no calibration required	Root length, surface area and volume; root tip count and location	C	Semiautomated	Any	msp, bmp, tif, pcx	Delta-T website	www.delta-t.co.uk/groups.html?group2005092301354
EZ-Rhizo	EZ-Rhizo enables the analysis of images of root growth in 2D (e.g. on agar plates). It permits root detection and measurements of multiple root architecture traits. It is a good tool for developmental and phenotypic descriptions of individual plants and genotypes and for root growth under varying nutritional and environmental conditions	Root length; insertion and insertion angle; number of branches; topological properties	F	Semiautomated	Arabidopsis	bmp	Armengaud et al. (2009), Armengaud (2009)	www.ez-rhizo.psrg.org.uk/

(continued)

Table 8.2 (continued)

Software	Brief descriptions	Root traits	Licence	Automation level	Plant requirement	Image requirements	Source	Website
GiA roots	GiA Roots (General Image Analysis of Roots) is capable of automating the large-scale analysis of root networks. It helps users easily quantify root structure	Root length, surface area and volume; number of branches; root depth; convex hull	F	Automated	Seedlings to mature plants	Any	Galkovskyi et al. (2012)	www.rootnet.biology.gatech.edu/giaroots/download/signup.php
GROWSCREEN-Root	GROWSCREEN-Root provides automatic analysis of root architecture. Images of whole root systems grown in agar-filled dishes are acquired with a CCD-camera. The key element of the software is the extraction of a tree model for a root system	Root length; insertion angle; number of branches	OD	Semiautomated	Any	Any	Nagel et al. (2009)	www.fz-juelich.de/ibg/ibg-2/EN/methods_jppc/GROWSCREEN_root/_node.html
Growth Explorer	Growth Explorer is designed for analysing root growth patterns from 2D time-series images, treating them as a spatio-temporal 3D image volume	Velocity profile	OD	Automated	Any	Any	Basu and Pal (2012)	www.plant-image-analysis.org/software/growthexplorer
IJRhizo	IJRhizo is an ImageJ macro for the batch processing of scanned images of root samples physically separated from soil by washing. It automatically generates root length and radius measurements	Root length and diameter; length of primary roots; total number and length of lateral roots; angle of branching	O&F	Automated	Any	jpg	Pierret et al. (2013)	www.plant-image-analysis.org/software/IJ_Rhizo

ImageJ	ImageJ is a public domain Java image-processing program. It can measure distances and angles and calculate area and pixel value statistics of user-defined selections. It supports standard image processing functions. Spatial calibration is available to provide real-world dimensional measurements in units	Root length; root area; distance between root nodes; branching angles	O&F	Semiautomated	Any	Any	Kimura et al. (1999), Tajima and Kato (2011)	rsb.info.nih.gov/ij/
Root System Analyser	Root System Analyser package analyses 2D images or image sequences of roots. It requires MATLAB to run	Root length and diameter; insertion and insertion angles; root tip count; root connectivity; inter-root distances; branching angles	F	Automated	Any	Any, segmented	Leitner et al. (2014)	www.csc.univie.ac.at/rootbox/rsa.html
RootDetection	RootDetection detects single-strand roots traces their paths and measures the resulting length automatically. It also provides tools for manually tracing features such as hypocotyls or side roots	Root length; tracing roots	F	Automated; semiautomated; manual	*Arabidopsis* seedlings	Any		www.labutils.de/rd.html
Rootfly	Rootfly is specifically designed for analysing minirhizotron images. It can track root development and determine root lifespan	Root length and diameter; colour; root initiation and death rates	O&F	Manual	Seedlings to mature plants, mini-rhizotron	jpg, bmp	Zeng et al. (2008)	www.ces.clemson.edu/~stb/rootfly/

(continued)

Table 8.2 (continued)

Software	Brief descriptions	Root traits	Licence	Automation level	Plant requirement	Image requirements	Source	Website
RootNav	RootNav facilitates reconstruction and quantification of root architecture. Root data can be exported and then analysed in bulk using the supplied viewer tool	Root length and count; convex hull; insertion and insertion angles	F	Semiautomated	Any	Any	Pound et al. (2013)	sourceforge.net/projects/rootnav/
RooTrak	RooTrak is designed for X-ray CT imaging data. It is able to adapt to changing root density estimates. It can extract a range of root architectural traits for the soil-grown roots	3D reconstruction of roots	F	Semiautomated	Mature, seedlings	Micro-CTimage sequence	Mairhoferet al. (2012)	sourceforge.net/projects/rootrak/
RootReader2D	RootReader2D is focusing on high-throughput analysis of total root length and selected root types of interest. The expended package performs lateral root counts for selected roots and has capabilities to work with the width-class groups and branching angles. It has been adapted to work roots systems that are grown in gellan gum, sand, agarose plate and paper pouch growth systems	Root length and depth; number of branches; topological properties	F	Semiautomated	Mature, seedlings	Tiff, jpg, png, bmp	Clark et al. (2013)	www.plantmineralnutrition.net/rootreader.htm

RootReader3D	RootReader3D software is designed to reconstruct and quantify 3D root system architecture descriptors from 2D rotational image sequences	Root length, diameter and surface area; root depth and volume; convex hull; number of branches; root orientation; insertion angles	OD	Automated	Seedlings to mature plants	Projection, any	Clark et al. (2011)	www.plantmineralnutrition.net/rootreader.htm
RootScape	RootScape is a landmark-based allometric method for rapid phenotyping of root system architecture	Root shape	F	Semiautomated	Arabidopsis	gif, tiff, jpg, png	Ristova et al. (2013)	www.atmosstudio.com/Rootscape
RootSnap!	The software has tracing enhancements to snap root tracing points to the centre of the root automatically. It can monitor root growth, disease, dynamics and behaviour over time and simplify mapping roots	Root length, diameter, surface area and volume	C	Semiautomated	Any	Any	Juraniec et al. (2014)	www.cid-inc.com/root-snap
RootTrace	RootTrace allows automatic and high-throughput measure of root length and curvature. It can trace the main root to the tip in every image in a time series. The software has been extended to count emerged lateral roots and to recover strongly curved and agravitropic roots	Root length; curvature; number of branches	O&F	Automated	Arabidopsis, seedlings	Time series	French et al. (2009)	sourceforge.net/projects/roottrace/

(continued)

Table 8.2 (continued)

Software	Brief descriptions	Root traits	Licence	Automation level	Plant requirement	Image requirements	Source	Website
RootView	The package is designed for working with images from minirhizotrons to study the growth and demography of fine roots and mycorrhizas: roots can be classified into different user-defined categories, their length and thickness are automatically measured from tracings, and root tips can be assigned to categories	Root length and diameter	F	Manual	Minirhizotron	jpg	Pedro Jos Aphalo, University of Helsinki	www.mv.helsinki.fi/aphalo/RootView.html
Skye	The software captures images at the click of a button from USB 'Plug & Play' video cameras or scanners. It has an automatic calibration function for setting up the pixel size and obtaining more accurate measurements	Root length and surface area; number of branches and root tips	C	Automated	Mature, seedlings	Any	Skye instruments	www.skyeinstruments.com/products/plant-analysis-systems/leaf-arearoot-length-systems/
SmartRoot	SmartRoot combines a vectorial representation of root objects with a powerful tracing algorithm which accommodates a wide range of image sources	Root length and diameter; insertion and insertion angles; root orientation; number of branches; topological properties	F	Semiautomated	Seedlings to mature plants	tiff, gif, jpeg, bmp	Lobet et al. (2011)	www.uclouvain.be/en-smartroot

	and qualities. It supports a sampling-based analysis of root system images. Requires ImageJ to run							
WinRHIZO	WinRHIZO is specifically designed for root measurements, including morphological, topological, architectural and colour analyses. The colour used to draw the root skeleton indicates into which diameter class the part of the root has been classified	Root length, diameter, surface area and volume; topological properties; colour	C	Automated	Seedlings to mature plants	tiff, jpg, png	Arsenault et al. (1995)	www.regentinstruments.com
WinRhizoTRON	The software measures roots and displays complete morphological information on screen. The distribution of root length, area, volume or number of tips is displayed as a function of diameter. It measures the real root diameter distribution rather than the average diameter	Root length, diameter, surface area and volume; topological properties; colour	C	Manual	Seedlings to mature plants rhizotron	tiff, jpg, png	Arsenault et al. (1995)	www.regentinstruments.com

Under 'Licence', *C* commercial, *F* free ware, *O&F* open and free ware, *OD* on demand

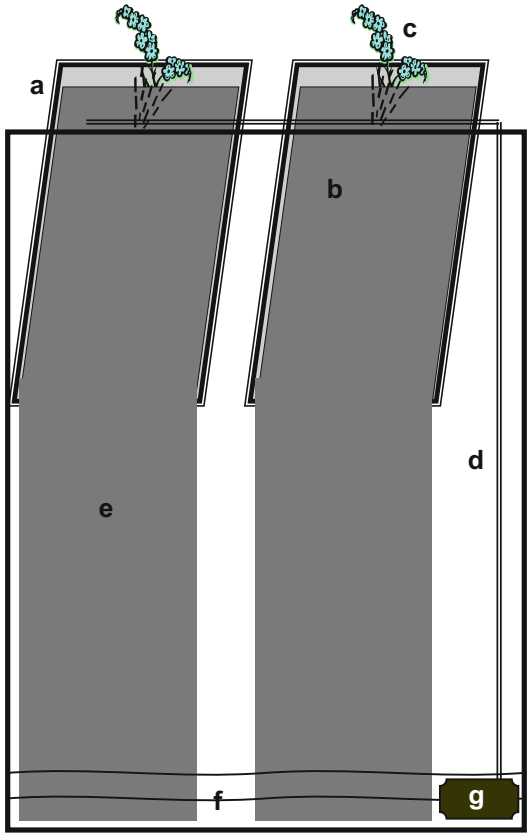

Fig. 8.3 Schematic presentation of the semi-hydroponic phenotyping system for two-dimensional, non-destructive measurements of root system architecture. *A* clear flat acrylic panel, *B* supporting cloth, *C* lupin plant, *D* 240-L bin, *E* extended cloth, *F* water or nutrient solution, *G* pump. Support framework not shown (Modified from Chen et al. 2011)

significantly reduces environmental stresses by optimising water supply with the equipped automatic irrigation system with a controller. Furthermore, it offers the opportunity to expose plants to abiotic stresses (e.g. acidity, salinity, drought, nutrient deficiency, elemental toxicity, etc.) whose evaluation under field conditions is usually quite difficult due to environmental variability affecting the intensity of the stress.

The semi-hydroponic/aeroponic system permits digital mapping of growth dynamics of taproots and lateral roots over time. This growing system overcomes the long-standing unsolved problem of phenotyping large sets of genotypes for rooting traits, which is particularly important for the identification of QTL and characterisation of molecular markers that may be useful in breeding. The semi-hydroponic phenotyping system was compared with soil-filled pot experiments, and the consistent ranking of root traits was produced for a range of genotypes of narrow-leafed lupin (Chen et al. 2011b).

Growth pouches developed from the hydroponic method were used in characterising root architecture traits such as growth angle and gravitropism of basal roots in common bean (*Phaseolus vulgaris*) related to phosphorus acquisition efficiency (Bonser et al. 1996; Liao et al. 2004). Hund et al. (2009b) improved the pouch system for rapid measurements of lateral root growth of maize. In this system, roots grew on the surface of blotting paper, thus facilitating the two-dimensional observation of root growth over time during the early days of root growth. However, phenotyping large sets of genotypes beyond very early growth stages using pouch systems remains problematic, particularly for QTL mapping studies.

8.3.2 Field

Even though laboratory/glasshouse phenotyping methods provide controlled environments, allow increased throughput and require fewer resources, they may not accurately reflect plant performance under field conditions. Nevertheless, significant associations between root traits of the seedlings grown under controlled conditions and those of the plants grown in the field were found in wheat (Mian et al. 1994; Richards 1996) and maize (Landi et al. 1998; Tuberosa et al. 2002b). However, it is challenging to extrapolate plant performance at the seedling stage when grown in artificial growth media to potential growth in the field (Iyer-Pascuzzi et al. 2010; Sanguineti et al. 2006). Therefore, high-throughput phenotyping in the field is needed to complement and validate studies in the controlled environments.

Field studies provide ground-truthing of plant growth in a particular environment, but phenotyping for root traits in the field conditions

is difficult because of the inability to visualise root systems in situ. Traditional methods of observing root system architecture including excavation (followed by root washing) are commonly used in studying roots in the soil environments (Gregory et al. 2003; Smit et al. 2000). Root systems are often manually excavated or augured for quantifying root length, biomass, distribution in soil and morphology and temporal variation of root growth. Excavation-related methods include excavations of (1) root system, (2) undisturbed cores or blocks of soil and (3) installed cores prefilled with root-free soil.

Shovelomics, an emerging term for a high-throughput phenotyping method using root excavation, was recently used for visual scoring of excavated root crowns to assess different root architecture traits of field-grown maize at flowering (Trachsel et al. 2011, 2013). On average, the total time required for excavation, soaking, rinsing and evaluation of root crowns was 10 min for silt–loam and 5 min for sandy soil. The root architectural traits assessed included the number of whorls occupied by brace roots, number of brace roots originating from whorl 1 to whorl 2, the branching density of brace roots and the number, angle and branching density of crown roots. Recently, we used shovelomics technique to evaluate genotypic variability in root traits of eight genotypes of narrow-leafed lupin in response to soil compaction and hardpan in the wheat belt of Western Australia (Chen et al. 2014). Application of shovelomics in other field-grown crop species requires further exploration.

Excavation methods are still commonly used in field studies today simply because of a lack of other reliable techniques. Although excavation techniques can be valuable, they are generally destructive, tedious and time consuming. One of the limitations of excavation-related methods is that they (1) often destroy the topology of the root system, leading to an underestimation of fine roots through breakage during excavation and washing, and (2) make it impossible to evaluate dynamics of root growth (Clark et al. 2011; Smit et al. 2000). Furthermore, root growth and architectural traits in soil environments are inevitably influenced by (1) soil heterogeneity (Lynch 1995) and (2) physical, chemical and biological interactions in the rhizosphere (Shaff et al. 2009; Ward et al. 2008).

To improve throughput in analysing excavated roots, image analysis methods coupled with data mining approaches have been developed to characterise root architecture. Transparent minirhizotron tubes can be installed vertically, horizontally or at various angles in the field (or in mesocosms) (Bates 1937). Roots that grow around the outside walls of the tubes can be imaged with cameras inserted down the tube length. Minirhizotrons allow the observation of root traits such as elongation rate, density, surface area, number and length at different soil depths throughout the growing season (Ao et al. 2010; Hendrick and Pregitzer 1992; Johnson et al. 2001). Recently, minirhizotrons were used to study root cortical aerenchyma in maize in response to suboptimal availability of soil nutrients, i.e. nitrogen, P and potassium (Postma and Lynch 2011), or water deficiency (Zhu et al. 2010). Because repeated observations can be made over time, minirhizotrons are particularly well suited for estimating root production and turnover (Johnson et al. 2001) as well as for estimating root biomass per unit of soil.

One limitation of minirhizotrons is that space may be created around the soil–tube interface that could influence root growth if the tubes are not installed properly. Furthermore, minirhizotrons only capture a fraction of the total root architecture. In this regard, they are better suited for measuring fine roots than coarse roots because fine roots are imaged more frequently and are more likely to be fully captured in images.

Traditional soil coring and trench profiling can be used as complementary techniques to minirhizotrons (Achat et al. 2008; Watt et al. 2005, 2008; Zhu et al. 2010). Like minirhizotrons, however, neither of these methods provides a full description of root system architecture, and both are tedious and time consuming (Vamerali et al. 1999).

A DNA-based method has been established to quantify changes in the root DNA concentration

in soil (Huang et al. 2013; Topp et al. 2013). It may provide fast and accurate measurements of root biomass in soil samples as a complementary method to root quantification.

8.4 Imaging and Analysis Platforms

8.4.1 Imaging Techniques

Root phenotyping approaches discussed above often require efficient imaging techniques, preferably in 3D. Several 3D imaging techniques have recently been used to non-destructively image root systems, including stereoscopics (e.g. Wulfsohn et al. 1999), neutron radiography (Oswald et al. 2008), magnetic resonance imaging (MRI; e.g. Rascher et al. 2011), ground-penetrating radar (e.g. Stover et al. 2007) and X-ray computed tomography (CT; e.g. Flavel et al. 2012).

Stereoscopic methods using plants grown in transparent media (Wulfsohn et al. 1999) enable visualisation of dynamics of root growth using RootViz FS (Phenotype Screening Corporation, Knoxville, TN, USA). Applications of this method are limited due to the need for manually rotating the microscope stage to see different parts of the root system and to image roots horizontally.

Neutron radiography (Oswald et al. 2008), magnetic resonance imaging (Asseng et al. 2000; Rascher et al. 2011), nuclear magnetic resonance (Jahnke et al. 2009; van der Weerd et al. 2001) and ground-penetrating radar (Stover et al. 2007) are used in field studies, but the current scale, resolution, throughput, accessibility and cost efficiency of these techniques limit their utility (Clark et al. 2011). Neutron radiography technique has the advantage in monitoring water distribution and root growth simultaneously, making it suitable for studying root–water relationships in soils (e.g. Oswald et al. 2008; Stingaciu et al. 2013). However, root images produced by neutron radiography are 2D and thus require specific image-analysing software packages (such as Root System Analyzer, Leitner et al. 2014) to recover root traits. Nuclear magnetic resonance is very sensitive to the type of media used for plant growth. Laser scanning of root systems (Fang et al. 2009) provides precise measurements, but requires relatively long imaging times and can be expensive.

Ground-penetrating radar and electrical resistivity imaging are low-resolution geophysical techniques that have been adapted for non-invasive imaging of roots in field-grown plants. Ground-penetrating radar uses pulses of high-frequency radio waves to image subsurface structures based on differences in their dielectric constants. It is rapid, but detection is generally limited to thick roots (at least 0.5 cm) at relatively shallow depths, depending on the soil type (e.g. dry, sandy soils are optimal). These limitations make ground-penetrating radar primarily useful for measuring root biomass of woody species (Stover et al. 2007).

Recent developments in X-ray CT (micro-scale CT, μCT) provide a breakthrough technology for non-invasively visualising root growth in soil (Flavel et al. 2012; Garbout et al. 2012; Mooney et al. 2012; Perret et al. 2007; Tray et al. 2010). Even though many papers over the past decades have concentrated on the method development from visualisation of roots in soil (e.g. Gregory et al. 2003) to automated segmentation of the whole root system architecture (e.g. Flavel et al. 2012), some of the most recent research is using X-ray CT to address fundamental questions regarding the functioning of the rhizosphere. Carminati et al. (2009) used X-ray CT to observe the dynamics of air gaps at a 90-μm resolution in the white lupin rhizosphere in response to wetting and drying cycles. Recent work of Aravena et al. (2013) measured compaction and provided new insights into soil–water uptake in sweet pea (*Lathyrus odoratus*) and sunflower (*Helianthus annuus*) using a 4.4-μm resolution CT technique.

Sophisticated image processing techniques, frequently based on the object-tracking methods, have demonstrated a great promise in measuring root traits of soil-grown plants at high resolution (Mooney et al. 2012). Using synchrotron

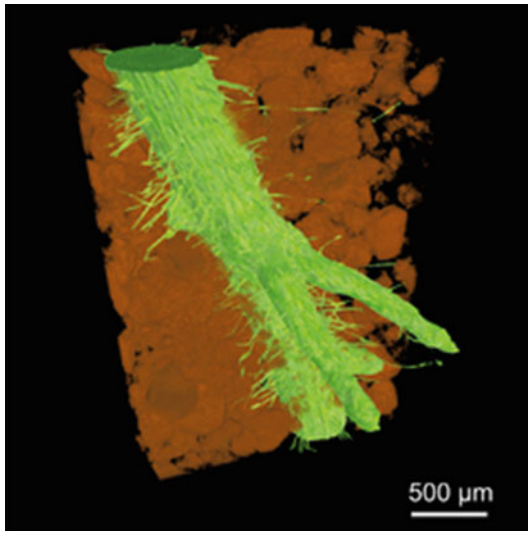

Fig. 8.4 Example image of X-ray computed tomography (CT) showing approx. 3-mm section of a seminal root of wheat (*Triticum aestivum*) (Keyes et al. 2013)

radiation X-ray tomographic microscopy (SRXTM) technique, Keyes et al. (2013) uncovered the 3D interactions of wheat root hairs in soil (Fig. 8.4), leading to the development of a model of phosphate uptake by root hairs based on the geometry of hairs and associated soil pores.

New X-ray CT-based root imaging approaches promise to complement and extend screening for root traits, potentially providing breeders with a 'deep phenotyping' capability (Flavel et al. 2012; Mooney et al. 2012). For example, crop root systems could be studied at high resolution and in 3D to reveal which architectural features might be most readily associated with water and nutrient uptake. The quality of 'region of interest' scans, i.e. zooming into a large sample and scanning a small volume at high resolution, has also recently improved.

The X-ray CT offers an elegant method of studying root growth non-destructively in situ, but would benefit from substantial hardware and software development to obtain high-resolution images of roots grown in relatively large containers. To that effect, a project recently funded by the European Research Council is attempting to improve CT hardware, software and genetic selection, offering a potential for enhanced in situ studies of root systems in the future.

8.4.2 Root Image-Analysing Software

High-throughput phenotyping platforms coupled to non-invasive root observation technologies acquire large numbers of root images. More than 30 different software tools are currently available analysing root system images (Table 8.2; Lobet et al. 2013).

General-purpose image-analysing software, such as WinRhizo and ImageJ, may be flexible enough to perform many specialised tasks. Pierret et al. (2013) confirmed the good performance of automated measurement of scanned root images using IJ_Rhizo in comparison with the commercial package WinRhizo.

A number of specifically designed image-analysing packages have also been developed for high-throughput quantification of root architecture traits. For example, there are several software packages for automating the analysis of root traits in minirhizotron images, including RootView, RooTracker, MR-RIPL and WinRhizoTRON. French et al. (2009) described the application of RootTrace software for high-throughput, automatic measurements of *Arabidopsis* seedling roots grown on agarose plates. The method combines a particle-filtering algorithm with a graph-based method to trace the centre line of a root. The package can quantify root length, curvature and stimulus response parameters such as onset of gravitropism, through tracing function. Leitner et al. (2014) developed a novel approach for recovering root architecture traits from 2D neutron radiography images based on image-analysing techniques in Root System Analyzer software. Information about these image-analysing programs and their applications are summarised in Table 8.2. Other useful data are available in Clark et al. (2013) and Lobet et al. (2013).

8.5 Root Models and Simulations

The complex interactions between root systems and their soil environment, and the difficulties associated with visualising and measuring these interactions, make studying the plant–soil continuum a challenge (Dunbabin et al. 2013). Current development of 3D root architectural models offers an excellent opportunity to characterise root function in soil, determine factors governing root–soil interactions and identify root parameters that underpin adaptation to a particular environment. Root models can be used to simulate (1) 3D and time-dynamic root architecture; (2) biological, physical and chemical processes occurring in soil; (3) scenarios beyond those directly observed; and (4) these scenarios in a dynamic environments that vary in time and space (De Dorlodot et al. 2007). Thus, by integrating rhizosphere and growth data, simulation and modelling studies are capable of linking predictive laboratory techniques with field studies, allowing researchers to strategically predict, evaluate and target beneficial root traits or genotypes for specific growth environments (De Dorlodot et al. 2007; Ho et al. 2004).

The development of structure-function root models and the features of six current root models have recently been reviewed in Dunbabin et al. (2013). These six root models are SimRoot (Lynch et al. 1997), SPACSYS (Bingham and Wu 2011; Wu et al. 2007), RootBox (Leitner et al. 2010), ROOTMAP (Diggle 1988a, b; Dunbabin et al. 2002b), RootTyp (Pagès et al. 2004) and R-SWMS (Somma et al. 1997). These models have been used for a wide range of root modelling studies (see Dunbabin et al. 2013).

ROOTMAP and SimRoot, the two simulation models that differ in the structure and functionality of modules, are being used to investigate various root–soil interactions in crops. ROOTMAP model combines the 3D growth and structure of root systems (Diggle 1988a, b) with root responses to spatial and temporal patterns of mineral nitrogen concentration in the environment (Dunbabin et al. 2001a, b) to produce an interactive model of root structure and function (Dunbabin et al. 2002b). So far, ROOTMAP was used to model (1) root growth of lupins, field peas and wheat and (2) uptake of water and nutrients (N and P) from soils with varying resistances to root growth and differing water and nutrient supplies at scales ranging from rhizosphere (Dunbabin et al. 2006) to field (Dunbabin et al. 2002a). The SimRoot model (Lynch et al. 1997) was previously used to select optimal root traits for phosphorus efficiency in beans, followed by selecting breeding lines and developing commercial cultivars that are now widely grown in Central and South America (Lynch and Brown 2001; Nielsen et al. 1998). Key strengths of SimRoot are (1) sophisticated routines for estimating carbon costs of various root structures and their efficiency in capturing P from soil (Lynch and Ho 2005; Nielsen et al. 1994), (2) use of fractal geometry to estimate 3D root growth from relatively easily measurable root parameters (Nielsen et al. 1997) and (3) capacity to represent changes in physiology and morphology along a root at high spatial resolution. Both models were recently used in studying lupin roots via parameterising with the root data acquired from the semi-hydroponic phenotyping system (Sect. 8.3.1; Chen et al. 2011a). Both models simulated root growth and responses to soil phosphorus in genotypes with contrasting root architecture under growth conditions similar to those of the glasshouse experiment (Chen et al. 2011b, 2013b).

It is anticipated that the structure-function root models will play an increasingly important role in the rhizosphere research, providing insights into the relationships among root architecture, morphology and functional efficiency (Dunbabin et al. 2013). With further development, root models have the potential to be used as an aid in crop breeding programmes by selecting root traits important for enhanced plant performance and grain yield in targeted environments.

8.6 Conclusion

Breeders, agronomists and other researchers recognise the significance of RSA to crop productivity. Increasing attention is being paid to searching for root traits conferring efficiency in resource acquisition and adaptation to edaphic stresses, particularly in drying soil environments. Various technologies are being developed for high-throughput phenotyping, non-invasive visualisation and accurate image analysis of root architecture traits. Together with recent developments of the structure-function simulation models, these advanced approaches will enhance our understanding of the relationship between root architecture and function and the complexity of root–soil interactions, leading to improved crop performance and productivity.

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Phenotyping for Problem Soils

9

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Abstract

Problem soils have serious physical and chemical constraints, impose stress on crops under cultivation and reduce its yield and productivity. Research efforts towards the development of tolerant cultivars for problem soils have gained all the advances from research areas of genomics but precision phenotyping still remains challenging. This chapter illustrates different phenotyping methods which have been used to screen genotypes against various problem soil conditions, discusses bottlenecks in the classical methods of phenotyping and exemplifies the application of high-throughput (HT) phenotyping in the current field of interest through red, green, blue (RGB) imaging, infrared thermography, chlorophyll fluorescence and hyperspectral imaging technologies. The HT phenotyping is a useful technique, and when it is empowered with other tools such as high-density linkage mapping and association mapping, it can accelerate the breeding process.

Keywords

Solis • Acidic • Alkaline • Nutrient deficient • Genetic variability • Precision screening

9.1 Introduction

Soil is the key environmental factor that affects plant growth and development. The properties of the soil are usually embedded in various definitions of soil fertility. Reuler and Prins (1993) defined soil fertility as the capacity of the soil to provide nutrients, water and oxygen to plants. Simply, we could define soil fertility as the capacity of the soil to support plant growth.

The concept of problem soils does not bring any concrete definition yet. According to Osman (2013), soils which impose limitation in crop cultivation and need special management practices are called problem soils. Having a severe limitation of certain soil physical constraints such as dryness, wetness, steepness and poor texture and chemical constraints such as

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acidity, salinity and sodicity, these soils demonstrate lowest priority for crop cultivation (Huq and Shoaib 2013; Osman 2013).

Problem soils affect plant growth and productivity not only due to the presence of high toxic minerals but also by limiting the supply of essential mineral nutrients (White and Greenwood 2013). Limited availability of macronutrients including nitrogen (N), phosphorus (P) and potassium (K) and micronutrients, namely, magnesium (Mg), iron (Fe), zinc (B) and boron (B), challenges crop cultivation in many regions (Mueller et al. 2012). Problem soils also undergo water-related stresses including restricted root growth through mechanical impedance in drying soils, limited porosity and waterlogging and affect crop cultivation (Hallett and Bengough 2013).

As a result of the rising population, depleting natural resources and unsustainable agronomic practices, agriculture faces tremendous challenges and it would be getting bigger further in the near future (Cobb et al. 2013). The Food and Agriculture Organization of the United Nations (FAO) predicts that world food production needs to be doubled to meet out the human demand by 2050 (http://www.fao.org). Since a large part of the good quality land has already been used for agriculture in the past, it is the right time to pay attention on increasing productivity on the lands where crop cultivation was in practice a while ago but relinquished by farmers very recently due to various soil-related problems (Wild 2003).

Many agronomic practices have been developed to address various soil-related problems. Conservation tillage and zero tillage practices have been successfully employed by farmers to cultivate crops in soils with more physical impediments such as erosion and waterlogged conditions. Application of mineral fertilisers has been increasing the yield potential of agricultural crops since the 1950s. But in other chemical constraints, for example, soil salinity problems, the farm management practices become unsustainable and necessitate huge efforts and investment. Eventually, plant-based strategies such as development of tolerant cultivars could provide possible alternative solutions to address this current issue (Abdalla et al. 2013; Hajkowicz and Young 2005; Llewellyn et al. 2012).

The presence of genetic variability for a particular trait of interest offers the scope for further improvement. In history, plant breeders either utilise the genetic variability for grain yield under specific target environments (George et al. 2014) or look for traits associated with grain yield; for instance, Na^+ exclusion is the key trait associated with salinity tolerance, and the selection of genotypes with low Na^+ accumulation favours salinity tolerance. However, the success of the breeding programme mainly depends on the availability of a reliable screening protocol that helps to differentiate the potential lines for a particular trait of interest for further selection and hybridisation.

Extensive research has been carried out during the past two decades on plant genomics in various crops. Advanced molecular marker technologies such as single nucleotide polymorphism (SNPs) markers have facilitated genomic selections by high-throughput genotyping at DNA level. However, the phenotyping remains a challenging task; genotype by environment interactions (GE) plays a significant role in the phenotypic data collected in the field as well as in greenhouses. Further, the subjective nature of the visual scoring of plant damage symptoms by classical phenotyping methods more frequently produces erratic results (Sirault et al. 2009).

Recent advances in the imaging technologies open up new opportunities for high-throughput (HT) phenotyping and screening of various biotic and abiotic stresses in plants. For instance, RGB images are useful to study changes in plant growth and health status under salt stress (Rajendran et al. 2009; Schilling et al. 2014) and B deficiency (Hayes et al. 2013), thermal images are useful to screen osmotic stress by diagnosing leaf water status and leaf temperature (Sirault et al. 2009) under water-stressed conditions, chlorophyll fluorescence images are quite useful to detect N deficiency (Antal et al. 2010; Donnini et al. 2013), and

hyperspectral images are useful to screen the genotypic differences for some micronutrient deficiencies (Shi et al. 2011, 2012a, b) in various crops.

This chapter provides an overview of the nature, causes and crop production constraints under problem soil conditions, assesses the importance of plant-based strategies in order to address the issue in current area of interest and reviews the genetic variability for plant tolerance to various problem soil conditions. Further, it demonstrates the range of classical phenotypic methods which have been employed to screen plant tolerance for various problem soil conditions and discusses the practical difficulties in classical phenotyping methods and its utilisation in the breeding programme. Finally, it highlights the applications of HT phenotyping methods by RGB imaging, infrared thermography, chlorophyll fluorescence and spectral imaging techniques and its utilisation to characterise genotypes for tolerance to various problem soil conditions.

9.2 Nature, Causes and Production Constraints in Problem Soils

This section discusses the nature, causes and production constraints in some of the major problem soils such as acidic, saline, sodic or alkaline, nutrient-deficient, contaminated or toxic and waterlogged or flooded or poorly drained soils as follows.

9.2.1 Acidic Soils

Soil acidification is the process of natural building up of hydrogen ions (H^+), also called protons, over time (Hajkowicz and Young 2005). The donor can be an acid from the acid rain such as nitric acid and sulfuric acid; sometimes many N compounds, in the form of fertiliser, also acidify soil over the long term. They produce nitrous and nitric acids when they get oxidised during nitrification process and favour the development of soil to be more acidic in nature (Hajkowicz and Young 2005). The degree of acidification is usually measured in terms of soil pH. Generally, the soil with pH <7 is declared as acidic, a pH of 7 is neutral and a pH >7 is alkaline (Slattery et al. 1999). In acid soils at pH <5.5, aluminium (Al) and manganese (Mn) become more soluble; they may become toxic as their concentration in the soil solution rises (Ring et al. 1993). Al inhibits root growth in most plants and induces calcium (Ca), phosphorus (P) and molybdenum (Mo) deficiencies in plants. It reduces microbial activity and causes poor nodulation in legumes (http://soilwater.com.au/bettersoils/module6/6_7.htm). Overall, soil acidity does not itself cause growth and yield reductions usually, but it is associated with increase of other toxic minerals and a decrease in the availability of plant nutrients and affects plant growth and productivity.

9.2.2 Saline Soils

Soil salinisation is the process of accumulating water-soluble salts such as chlorides (Cl^-) and sulphates (SO_4^{2-}) of sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) and to a lesser extent of salts of carbonates (CO_3^{2-}) and bicarbonates (HCO_3^-) on the soil surface (Abrol et al. 1988; Rengasamy 2006). Weathering of parental rocks and deposition of oceanic salts (by wind and water) cause the development of saline soils in a natural way. Besides, the process of anthropedogenesis (supported by man-made activities), for instance, irrigating the farmland with poor-quality water, improper drainage structures and chemical pollution, also leads to the development of saline soils in some agricultural lands (FAO 2005). It is estimated that about 800 million ha of the world area is affected by soil salinity, which consists of about 6 % of the global land area (Munns and Tester 2008). Saline soils are characterised by the concentration of soluble salts in the soil solution (Munns and Tester 2008). It possesses the electrical conductivity of saturation extract (EC_e) of >4 days/m (approximately 40 mM), the exchangeable sodium

percentage (ESP) of >15 % and the soil pH <8.5 (Abrol et al. 1988).

Soil salinity induces osmotic and ionic stresses in plants (Munns et al. 1995). Osmotic stress affects seed germination and seedling emergence rate (Sayar et al. 2010) and shoot growth and reduces the number of tillers and leaf number in cereals (De Costa et al. 2007; Harris et al. 2010; Nuttall et al. 2006). Severe soil saline conditions cause permanent wilting of plants (Munns et al. 1995; Rengasamy 2006). The high concentrations of ions such as Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, HCO_3^-, NO_3^- and K^+ in the soil solution cause ion-specific toxicities (Bernstein 1975). Particularly, Na^+ is toxic to most of the annual field crops, whereas Cl^- is toxic to perennial horticultural crops. The Na^+ toxicity develops with the symptoms of marginal chlorosis and necrosis in the leaf blade, followed by premature leaf senescence and complete plant death (Tester and Davenport 2003).

9.2.3 Sodic/Alkaline Soils

Sodic soils are commonly referred to soils which have salts of the Cl^-, SO_4^{2-}, HCO_3^- and CO_3^{2-} of mainly Na^+ (Abrol et al. 1988). It is also known as alkaline soils. Sodic soils own poor soil structure particularly under dry environments. They appear black in colour and possess hard calcareous pan at 0.5–1 m depth below from the soil surface. Sodic soils are characterised by pH > 8.5 with the ESP >15 % and EC_e of <4 dS/m (Abrol et al. 1988). The poor structure of sodic soil creates mechanical impedance for roots to grow and penetrate inside the soil column under dry condition (Masle and Passioura 1987). The low infiltration capacity of sodic soil often causes slow water penetration and distribution in the soil column and allows water stagnation in the field (Oster and Jayawardane 1998). Further, the Na^+ in the soil particle often replaces Ca^{2+} and develops Na^+-induced Ca^{2+} deficiencies (Adcock et al. 2001; Ehret et al. 1990). They also induce CO_3^{2-} and HCO_3^- toxicities and K, Fe, Mn, Mg, Cu, Zn and P deficiencies in various crops (Naidu and Rengasamy 1993).

9.2.4 Nutrient-Deficient Soils

Nutrient deficiencies limit crop production in all types of soil around the world (Arnon 1954). A mineral is considered as a nutrient when without it plants cannot complete its life cycle. A total of 16 minerals are identified as essential nutrients for plant growth and development. It includes carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo) and chlorine (Cl). Except C, plants uptake all minerals from the soil solution, whereas C enters from the atmosphere, in the form of carbon dioxide (CO_2).

Based on the amount of requirements in the plant metabolic activities, nutrients are categorised into two groups such as macro and micronutrients. The minerals, namely, N, P, K, Ca, Mg and S, are grouped as macronutrients; their requirement ranges between 1 and 150 g per kg of plant dry matter. On the other hand, Fe, Zn, Mn, Cu, B, Mo and Cl are classified as micronutrients because these are required at a lesser rate than the previous group, 0.1–100 mg/kg of plant dry matter (Marschner 1997). For normal growth and function, plants require all these nutrients in balanced proportions. Any deviation from the above required quantities affects plant growth and health and causes nutritional disorders; for example, P deficiency in maize causes reddened tip with stunted plant growth (Gong et al. 2011), Mg deficiency in broad bean causes interveinal chlorosis (Hariadi and Shabala 2004a), Fe deficiency in soybean results in chlorosis (Norvell and Adams 2006), Zn deficiency in rice results in leaf bronzing (Wang et al. 2008; Wissuwa et al. 2006), and B deficiency in lentil causes leaf chlorosis (Srivastava et al. 2000). However, the continuous shortage of a nutrient or nutrients causes complete plant death.

9.2.5 Contaminated/Toxic Soils

A mineral becomes a toxin when plant uptakes higher than the optimum level of requirement. For example, Fe is an essential micronutrient, but the high concentration of Fe^{2+} in the soil solution causes leaf bronzing, necrotic rust leaf spots on the leaf tips, stained leaf edges, stunted shoot growth and poor root development, for instance, in rice (Elec et al. 2013; Sahrawat 2004). In fact, Fe^{2+} toxicity is one of the major problems of rice cultivation under wetland conditions.

Similarly, B toxicity is one of the most important problems of crop cultivation in the arid and semiarid areas all over the world. B toxicity occurs through natural process from marine evaporates and marine argillaceous sediments (Nable et al. 1997). Occasionally, the irrigation water contaminated by wastes from surface mining, fly ash and industrial chemicals increases the soil B availability and becomes toxic to plants. The symptoms of B toxicity develop by yellowing of leaflets of the lower leaf followed by the necrosis progressing towards the base of leaflets and leaf margins. Under severe stress, it causes dropping of leaflets in lentil; it begins from the older leaf at the bottom to the younger leaf at the top (Yau and Erskine 2000).

Besides, as described in Sect. 9.2.1, Al and Mn become toxic under acid soil conditions (Ring et al. 1993), and the groundwater contaminated by arsenic (As) and the use of As-contaminated groundwater for irrigation led to As toxicity. Arsenisation of soil in the Gangetic alluvium of Bangladesh is one good instance (Huq and Shoaib 2013).

9.2.6 Waterlogged/Flooded/Poorly Drained Soils

Waterlogging is a condition in which plants get very limited supply of oxygen with other notorious gases such as methane, nitrogen, etc., and affects crop cultivation (Huq and Shoaib 2013). Waterlogging conditions could arise due to the poor internal capillary drainage or high clay composition of soils. Otherwise, cropping systems such as rice followed by a pulse often develop waterlogging condition to the pulse crop grown after rice in some regions. The symptoms of waterlogging stress appear as leaf chlorosis, necrosis, stunting, defoliation and plant death (Cornelious et al. 2005). Waterlogging causes major yield reductions in wheat (Saqib et al. 2013), maize (Tripathi et al. 2003) and soybean (Cornelious et al. 2005).

9.3 Importance of Plant-Based Strategies to Address Problem Soils

Sustainable management of farmlands coupled with the development of tolerant varieties for particular problem soil conditions is one of the solutions to achieve necessary increase in crop productivity and hence the future food security (Bakker et al. 2012; Frison et al. 2011). Application of soil amendments including lime, organic manures, farmyard manures and green manures and rotation of land with grazing paddocks are useful practices to reclaim acid soil conditions (Bal 2001). On the other hand, plantation of deep-rooted perennials, gypsum application, seed priming and foliar application of growth hormones (Rengasamy 2002) are useful to address the soil salinity and sodicity issues. However, the impact of these agronomic practices on crop production is influenced by many socio-economic factors. Firstly, it needs amendments at huge quantities and can help to improve the conditions of the top soil only. Secondly, it takes more efforts, for example, plantation of deep-rooted perennials is also a difficult option; it is hard to keep the trees survived under salt-affected soils (Chhabra 1996). Thirdly, it is a long-term solution and takes time to see the results; certain treatments need repeated application continuously for a certain period. Finally, it all ends up with low benefit to cost ratios to the farming community (Hajkowicz and Young 2005). Eventually, the development of tolerant crop cultivars becomes a potential alternative

solution to address the problem of soil acidity and salinity.

In other cases, mineral fertilisation, the agronomic practice to correct soil nutrient deficiencies, demands high market price with the substantial damage to the environment (Stewart et al. 2005). In the last 40 years, the amount of synthetic nitrogen (N) applied to crops has risen dramatically, from 12 to 104 Tg⁄year, resulting in significant increases in yield but with considerable impacts on the environment such as the development of algal blooms (eutrophication), depletion of ozone in the atmosphere due to emission of greenhouse gases and very much influenced global warming. In fact, much of the N added to the soil becomes wastes, with an average of only 30 %–50 % being taken up by the plant, while the remnant being lost as a surface run-off (Mulvaney et al. 2009). We need to develop crop cultivars which have high N use efficiency (NUE) in terms of N uptake efficiency (NUpE) and N utilisation efficiency (NUtE). In this way, we can reduce the economic and environmental loss and achieve sustainable agricultural production in the near future.

9.4 Occurrence of Genetic Variability for Problem Soils

Occurrence of natural variation for the trait of interest is the key to develop such crop varieties (Koornneef et al. 2004). The presence of genetic variability for tolerance to acidity (Camargo et al. 1995; Foy 1996), salinity (Munns et al. 2000; Munns and James 2003), sodicity (Rao et al. 2008; Singh et al. 2002), toxic soils (Hasnain et al. 2011; Yau and Erskine 2000), nutrient use efficiency (Parentoni et al. 2010; Zhang et al. 2009) and waterlogging tolerance (Cornelious et al. 2005; Saqib et al. 2013) has been confirmed across different crops at both inter- and intraspecies levels. Past studies identified a wide range of variations to the corresponding soil condition based on traits including shoot growth, symptoms of toxicities and deficiencies and various physiological parameters such as leaf elongation rate, relative growth rate, tissue ion concentrations, etc., and by comparing differential response of genotypes in terms of plant biomass and grain yield under low fertile and low input soil conditions with the normal soil conditions (Table 9.1). These identified sources of variability have already been used to develop tolerance cultivars, for instance, the genetic variation in *Aegilops tauschii* for salinity tolerance was useful to develop synthetic hexaploids of bread wheat with improved salinity tolerance (Schachtman et al. 1992) through conventional breeding approach. On the other hand, marker-assisted selection has facilitated to improve salinity tolerance of wheat cultivars, and those cultivars can yield more than 25 % in the saline soils in Australia (James et al. 2011).

9.5 Rationale for Appropriate Phenotyping Methodology

Physiologists and breeders might consider using potential variations to develop cultivars for problem soils. However, the breeder should know the genetic control of the trait of interest and its gene action in order to choose suitable breeding method for the given trait of interest. If the trait is controlled by a dominant gene, it suggests development of hybrids in order to gain more in the cross-pollinated crops. On the other hand, if it is an additive gene action, the pedigree selection is suitable to improve those traits in self-pollinated crops.

Extensive research has been done to understand the variability for the traits related to problem soil conditions. In most of the studies, the genetic control of the tolerance mechanisms is questioned, and it seriously limits the effort of plant breeders to develop efficient breeding strategies to develop tolerant varieties. Recent reports suggest that the genetic control of tolerance to these problem soils is inherited by polygenes which involve the function of many genes with small individual effect and the expression of these traits is highly subjected to the environmental conditions. In one exemption, the monogenic inheritance was observed for Al

Table 9.1 Classical phenotyping methods used to characterise plants grown under various problem soil conditions

Nature of the soil problem	Screening technique/ criteria	Crops/plant species	Growth conditions	Parameters used	References
Acidity	Root regrowth method	Barley	Nutrient solution culture in glasshouse	Root recovery	Nawrot et al. (2001), Choudhary et al. (2011)
	Haematoxylin root tip staining method	Pigeon pea	Nutrient solution culture in glasshouse	Root tip damage	Nawrot et al. (2001), Choudhary et al. (2011)
	Soil bioassays	Wheat	Pot culture in glasshouse	Relative shoot and root dry weight	Foy (1996)
	Yield-based assays	Wheat	Field experimentation	Grain yield	Camargo et al. (1995)
Salinity	Physiological parameters	Wheat	Nutrient solution culture in glasshouse	Leaf elongation rate	Munns and James (2003)
		Wheat, barley and triticale	Nutrient solution culture in glasshouse	Leaf area expansion rate	Rawson et al. (1988)
		Rice and wheat	Nutrient solution culture in glasshouse	Relative growth rate	Lutts et al. (1996), James et al. (2008)
		Sugar beet	Nutrient solution culture in glasshouse	Relative water content	Ghoulamet al. (2002)
		Wheat	Nutrient solution culture in glasshouse	Stomatal conductance	James et al. (2008)
		Rice	Nutrient solution culture in glasshouse	Osmotic potential	Lutts et al. (1996)
		Wheat	Nutrient solution culture in glasshouse	Na^+ exclusion	Munns and James (2003), Shavrukov et al. (2009)
		Wheat	Nutrient solution culture in glasshouse	K^+/Na^+ discrimination	Munns and James (2003)
		Wheat	Nutrient solution culture in glasshouse	Symptoms of salt stress	Munns and James (2003)
	Salinity tolerance indices	*Lycopersicon* species	Petri-dish culturing	Germination rate	Foolad and Lin (1997)
		Halophytic plant species	Petri-dish culturing	Seedling survival rate	Ashkan and Moemeni (2013)
		Tomato	NSC^{15} in greenhouse	Plant height	Caro et al. (1991)
		Wheat	Nutrient solution culture in glasshouse	Shoot biomass	Munns and James (2003), Genc et al. (2007)
		Wheat	Field experimentation	Grain yield	Houshmand et al. (2005)
		Wheat	Field experimentation	Tissue Na^+ concentrations	El-Hendawy et al. (2009)
Alkalinity/ sodicity	Sodicity damage index	Rice	Nutrient solution culture in glasshouse	Symptoms of sodic sensitivity	Singh et al. (2002)
	Agronomical parameters	Rice	Field experimentation	Grain yield and yield components	Rao et al. (2008)

(continued)

Table 9.1 (continued)

Nature of the soil problem	Screening technique/ criteria	Crops/plant species	Growth conditions	Parameters used	References
Nutrient deficiencies					
N	Morpho-physiological parameters	Barley	Nutrient solution culture in glasshouse	Chlorophyll content	Hoffmann et al. (2012)
		Maize	Field experimentation	Grain yield and yield components, tissue N concentrations	Cirilo et al. (2009)
P	Plant damage assessment	Maize	Nutrient solution culture and pot culture in glasshouse	Symptoms of P deficiency	Gong et al. (2011)
	Physiological parameters	Soybean	Pot culture in outdoors	Relative growth rate and leaf elongation rate	Qiu and Israel (1994)
K	Physiological parameters	Cotton	Nutrient solution culture in glasshouse	Leaf elongation rate, leaf water potential	Gerardeaux et al. (2010)
	K uptake kinetics	Tomato	Nutrient solution culture in glasshouse	Tissue K concentrations	Pujos and Morard (1997)
Mg	Plant damage assessment	Broad beans	Soil hydroponic flooding system	Symptoms of Mg deficiency	Hariadi and Shabala (2004a)
	Physiological parameters	Broad beans	Nutrient solution culture in glasshouse	Co_2 assimilation, transpiration rate	Hariadi and Shabala (2004b)
Fe	Plant damage assessment	Soybean	Nutrient solution culture in glasshouse	Symptoms of Fe deficiency and leaf Fe concentrations	Norvell and Adams (2006)
	Fe stress tolerance indicator	Wheat	Field experimentation	Grain yield	Sadrarhami et al. (2010)
Zn	Plant damage assessment	Rice	Nutrient solution culture in growth chamber and field experimentation	Symptoms of Zn deficiency and shoot Zn concentrations	Wissuwa et al. (2006), Wang et al. (2008)
	Zn efficiency ratio	Wheat	Pot culture in greenhouse	Shoot biomass	Cakmak et al. (2001)
B	Grain set index	Barley	Pot culture and field experimentation	Number of spikes per plant, spikelets/spike	Jamjod and Rerkasem (1999)
	Plant damage assessment	Lentil	Field experimentation	Symptoms of B deficiency	Srivastava et al. (2000)
Nutrient use efficiency					
Nitrogen use efficiency (NUE)	Agro-biochemical parameters	Barley	Nutrient solution culture and pot culture in growth chamber along with field experimentation	Seed yield and metabolite levels	Beatty et al. (2010)
	Grain quality parameters	Wheat	Field experimentation	Grain yield and grain protein content	Anderson and Hoyle (1999)

(continued)

Table 9.1 (continued)

Nature of the soil problem	Screening technique/ criteria	Crops/plant species	Growth conditions	Parameters used	References
Phosphorus use efficiency (PUE)	Morphological parameters	Tomato	Nutrient solution culture in glasshouse	Plant biomass and root characters	da Silva and Maluf (2012)
Potassium K internal use efficiency (KIUE)	Agro-physiological parameters	Rice	Nutrient solution culture and field experimentation	Plant biomass, grain yield and tissue K concentrations	Yang et al. (2003)
Nutrient toxicities					
Fe	Plant damage assessment	Rice	Nutrient solution culture in greenhouse	Symptoms of Fe toxicity	Elec et al. (2013)
B	Seedling assays	Wheat	Filter paper technique	Root length	Chantachume et al. (1995)
		Mung bean	Petri-dish culture	Germination percentage	Hasnain et al. (2011)
	Agro-physiological parameters	Mung bean	Pot culture in glasshouse	Symptoms of B toxicity	Hasnain et al. (2011)
		Lentil	Pot culture in greenhouse	Symptoms of B toxicity	Yau and Erskine (2000)
		Brassica rapa	Nutrient solution culture in glasshouse	Shoot boron concentrations	Kaur et al. (2006)
Waterlogging	Plant damage assessment	Soybean	Field experimentation	Symptoms of waterlogging	Cornelious et al. (2005)
	Yield-based assays	Wheat	Nutrient solution culture	Plant biomass and grain yield	Saqib et al. (2013)
	Agro-physiological parameters	Maize	Field experimentation	Days to 50 % silking, plant height, canopy temperature, transpiration rate	Tripathi et al. (2003)

tolerance in wheat, but in barley and maize, it has been confirmed as a polygenic trait with additive gene actions (de Almeida et al. 2002; Ferreira et al. 2006; Minella and Sorrells 1997). Genotype × environment (GE) interaction causes difficulty on phenotypic selection of traits with polygenic inheritance (Cobb et al. 2013). Besides GE interactions, other physiological and genetic interactions between physiological pathways and genes controlling various component traits also mask the phenotype. Development of reliable phenotyping technique that can accurately examine these interactions and distinguish genotypes for these differences could help to develop a successful tolerant cultivar.

9.6 Classical Phenotyping Methods (from Glasshouse to Field)

Classical phenotyping methods usually involve data collection in shoots and roots of plants grown under stressed conditions. It quantifies the genotypic differences of plant characters including plant biomass (Genc et al. 2007), grain yield (Houshmand et al. 2005), yield components (El-Hendawy et al. 2009), plant health (Hasnain et al. 2011) and certain morphological (Souza et al. 2000) and physiological (James et al. 2008) parameters. It can be done under controlled (lab/glasshouse/greenhouse/growth chamber/phytotron) (Ashkan and

Moemeni 2013; Hoffmann et al. 2012; Khabaz-Saberi et al. 2010) as well as in field environment (El-Hendawy et al. 2009). In the controlled conditions, phenotyping is done in petri plate culture (Ashkan and Moemeni 2013; Foolad and Lin 1997), filter paper technique (Chantachume et al. 1995), hydroponic nutrient culture (Choudhary et al. 2011), supported hydroponic nutrient culture (James et al. 2008; Shavrukov et al. 2009), semi-hydroponic nutrient culture (Caro et al. 1991) and soil hydroponic flooding system (Hariadi and Shabala 2004a). In these methods, plant health is measured by visual scoring of symptoms in Zadoks scale (Khabaz-Saberi et al. 2010; Yau and Erskine 2000). Development of stress tolerance indices and ranking of genotypes based on their germination rate, seedling survival rate, plant height, shoot biomass, yield and yield components is a common practice (Ashkan and Moemeni 2013; El-Hendawy et al. 2007; El-Hendawy et al. 2009; Genc et al. 2007). The classical phenotyping methods are helpful to screen and identify genotypic differences for a wide range of problem soil conditions across different crop species but to a certain extent. The list of classical phenotyping methods used to phenotype and screen genotypes for tolerance to various problem soil conditions is provided in Table 9.1.

However, there are several factors affecting the efficiency of classical phenotyping methods. Some of the classical phenotyping methods are expensive and require a huge amount of labour and time (Sirault et al. 2009). Hariadi and Shabala (2004a) revealed leaf Mg nutrient analysis as an efficient way to study Mg deficiency in broad beans. But at the same time, it was declared as an expensive and time-consuming technique to screen large number of genotypes for Mg deficiency. It follows destructive approach to estimate crop tolerance. For example, leaf sampling and harvesting of whole plant shoot during the time of experimentation are widely followed by researchers as a part of salinity tolerance screening (Munns et al. 1995; Munns and James 2003). In this case, the same plant cannot be further examined once it is disturbed or harvested completely. Moreover, the classical methods mainly focus on relative changes in the physiological parameters including leaf elongation rate, leaf area expansion rate, relative growth rate, relative water content, etc., rather than whole plant response under stressed environment (Table 9.1). Hasegawa et al. (2000) opine that no single physiological observation can contribute the whole plant tolerance to various abiotic stresses; hence, researchers have to consider measuring the changes in the whole plant response rather than the individual physiological observations.

Importantly, quantification of symptoms of nutrient deficiencies and toxicities by scoring human eye frequently produces imprecise and unrepeatable results. The subjective nature of visual scoring coupled with the heterogeneity of the field environment often produces inconsistent results (Greenway and Munns 1980; Shannon 1985). In addition, symptoms of some nutrient deficiencies often get confused with other nutrient deficiencies, plant damages caused by pests, diseases and other related stresses. Toxicity of Mo or Se is similar to P deficiency (Bennett 1993); Fe deficiency in mango is similar to chloride toxicity (Xu et al. 2000). As a final point, it is hard to accurately phenotype some specific plant traits, for instance, root characteristics of plant growing under various soil-related problems through classical screening methods (Fleury et al. 2010; Richards et al. 2010; Zhu et al. 2011). According to Roy et al. (2011), root phenotyping needs to be considered to quantify Al tolerance, B tolerance, nitrogen deficiency and phosphate deficiency in various crop species.

9.7 High-Throughput (HT) Phenotyping Technologies (from Glasshouse to Field)

Recent progresses in the non-destructive imaging technology have allowed the researchers to monitor the changes in the growth, health and physiological status of whole plant response over time, non-destructively. Here these advanced

phenotyping technologies have been discussed in relation to phenotyping the traits associated with various soil problems. In fact, images can capture the large amounts of phenotypic information of plants being photographed over time. Particularly, in plant biology, images from electromagnetic waves are quite useful in many research areas to know the things which cannot be distinguishable by the human eye (Umbaugh 2005). For instance, images taken using visible light (RGB) infrared rays and chlorophyll fluorescence are quite useful to detect changes in morphological and physiological status of the plants grown under stressed environment and detect nutritional deficiency tolerance at early stages of plant ontogeny. It is rapid, consumes less labour and time and produces reliable results. It is objective and helps to obtain quantitative data of symptoms of plant damages for further QTL analysis. The following sections discuss some major applications of RGB imaging, infrared thermography, chlorophyll fluorescence and spectral imaging in the current field of interest.

9.7.1 RGB Imaging

It is the simplest and precise way of quantifying subtle changes in the growth and health status of plant shoots (projected shoot area) over time (Harris et al. 2010; Rajendran et al. 2009). It uses the visible range of electromagnetic spectrum (400–700 nm) with the help of CCD (charge-coupled device) cameras to obtain images of the plants from different angles (Arvidsson et al. 2011; Rajendran et al. 2009). With the help of an image analysing software, the images are processed to extract phenotypic information such as plant colour and some morphological parameters. After image analysis, the colour-classified image can provide information about the healthy (green), senescing/chlorotic (yellow) and senesced/necrotic (brown) parts of the leaf which is helpful to differentiate tolerant and sensitive cultivars, for example, to salt stress (Rajendran et al. 2009) and boron toxicity (Hayes et al. 2013; Schnurbusch et al. 2010). The calculated projected shoot area from these colour images has shown positive and significant correlations with the shoot biomass taken, immediately after the destructive harvest in barley and wheat (Golzarian et al. 2011; Harris et al. 2010; Rajendran et al. 2009). By providing the details of projected shoot area, it is useful to monitor changes in the growth rate of plants grown under stressed environments (Rajendran et al. 2009; Schilling et al. 2014).

There are many commercial RGB image-capturing tools available to obtain dynamic and spatially distinct parameters of plants grown under stressed environment. For instance, the LemnaTec Scanalyzer was useful to monitor the changes in the growth and health status of plants grown under salt stressed conditions non-destructively over time. Rajendran et al. (2009) developed a high-throughput salinity tolerance screening protocol that dissects the whole plant salinity tolerance into three major components such as Na^+ exclusion, osmotic tolerance and tissue tolerance. The tolerance indices developed using this scanalyzer were used to screen the variability in genotypes having different combinations of each of these three major components of salinity tolerance for further genetic studies.

Further, the RGB imaging was found useful to develop green vegetation indices of durum wheats grown under stressed environments in the field (Casadesus et al. 2007). It was also quite useful to quantify B toxicity in wheat and barley (Hayes et al. 2013; Schnurbusch et al. 2010), macronutrients (N, P and K) deficiencies in legumes (Wiwart et al. 2009) and N deficiency in barley (Pagola et al. 2009).

In general, RGB imaging is a robust, rapid and efficient method to phenotype plants for growth and health status grown under stressed conditions. Sometimes, the overlapping of leaves in plants under specific doses of treatments affects the relationship between projected shoot areas with shoot biomass and produces unrepeatable results (Golzarian et al. 2011). The background soil noise also often affects colour

classifications, which necessitates sound technical skills to do image analysis. Moreover, it provides only limited information about the physiological status of the plants (Fiorani and Schurr 2013).

9.7.2 Infrared Thermography

Quite often, breeders use variation in the canopy temperature as one of the selection criteria for tolerance to drought, and selection for canopy temperature correlates well to the grain yield (Fischer et al. 1998; Reynolds et al. 1999). The infrared thermography (3–14 μm) has been useful to study canopy temperature and the leaf water status of the plants grown under water-stressed environment (Jones et al. 2009; Munns et al. 2010). Plants growing under various problem soil conditions demonstrate similar physiological responses to drought stress, for instance, the closure of stomata and reduction in the photosynthetic area; it is possible to utilise the benefits of infrared thermography to phenotype plants grown under various problem soil conditions (Sirault et al. 2009).

It distinguishes genotypes based on the occurrence of stomatal conductance and hence the canopy temperature. Tolerant genotypes usually have high rate of stomatal conductance and possess cooler leaves than the sensitive ones (Berger et al. 2010). As the GE interactions influence the results under field conditions, it is used to detect genotypic differences in the stomatal conductance and transpiration rate of genotypes grown under controlled environments rather than in the field environment (Berger et al. 2010; Furbank and Tester 2011). However, the physics of the heat flux is highly variable and complicates the measurement in many times (Fiorani and Schurr 2013). Frequently, a combination of RGB images with infrared thermography is useful to study leaf orientation, canopy structure and canopy temperature of plants grown under stressed environments (Leinonen and Jones 2004).

9.7.3 Chlorophyll Fluorescence Imaging

Chlorophyll fluorescence has a wide application in high-throughput plant phenotyping from lab to field studies. It is found useful to measure the photosynthetic efficiency, the electron transport rate and the extent of non-photochemical quenching. The parameters of photochemical and non-photochemical quenching coefficients are used to study Mg deficiency in broad beans (Hariadi and Shabala 2004b). It is also useful to detect N deficiency in common bean (Antal et al. 2010) and maize (Lu and Zhang 2000), Fe deficiency in cucumber (Donnini et al. 2013), and NUE in maize (Corp et al. 2003). Most of the time, portable instruments used to measure photosynthetic status of plants create difficulties in terms of robustness, reproducibility and data analysis (Fiorani and Schurr 2013). Along with RGB imaging, chlorophyll fluorescence can provide information about the leaf area, growth rate and leaf senescence. Hence, the interpretation of results from both RGB images and fluorescence images could be more efficient to do early detection of nutrient deficiencies and toxicities and to quantify plant damages.

9.7.4 Hyperspectral Imaging

Hyperspectral imaging utilises the wavelength from 400 to 2500 nm, which falls between the spectral range of visible and near-infrared (NIR) regions. It has a major application in remote sensing (Kokaly et al. 2009). Hyperspectral reflectance measurements are useful to identify waveband signatures that help to indicate plant stress levels. It is useful to determine reflectance vegetation indices and helps to estimate biomass, healthiness, pigment composition, photosynthetic status, leaf thickness, growth habit and relative water content (Berger et al. 2010; Fiorani and Schurr 2013). In the current field of research, it is used to detect deficiencies caused by N (Shi et al. 2011, 2012a), P (Shi et al. 2012b) and Ca (Li et al. 2005) in various crops. NIR spectral

imaging needs extensive calibration before usage. Once calibrated, it facilitates non-destructive quantification of water content, protein content and other related compounds for large-scale phenotyping.

9.8 Automated Greenhouse (AGH) Facilities

All these high-throughput plant imaging technologies often necessitate highly automatic and rapid imaging of plants grown under controlled environments. Greenhouse facilities set up with the conveyor belts for delivery of plants to the imaging system, watering and weighing of plants in an automated way would help to do more precision fast phenotyping. Such greenhouse facilities have already been used to phenotype plant traits including plant height, width, area and biomass, chlorophyll, anthocyanin and foliar water content in corn, soybean and cotton (Hyundae et al. 2014). It is also used to detect deficiencies of Ca (Story et al. 2010) and Mg (Chaerle et al. 2007) in various crops. The AGH facilities are now available at CropDesign in Belgium (http://www.cropdesign.com/general.php), Leibniz Institute of Plant Genetics and Crop Plant Research-IPK in Germany (http://www.ipk-gatersleben.de/) and Australian Plant Phenomics Facility in Australia (http://www.plantphenomics.org.au/). They are often useful to do screening in germplasm and breeding populations to identify QTL and candidate gene(s) under controlled conditions; later these results can be validated in the well-planned field experimentation.

9.9 Concluding Remarks

The HT phenotyping technologies are useful to study the phenotypic changes in the growth, health and morphological changes of plants grown under different problem soils. The advantages of HT phenotyping are well recognised by breeders, physiologists and soil scientists involved in the current field of interest. Along with the advances in plant genomic research area, recent developments in plant phenomics would help to do more precise measurement of plant traits, identify new plant traits and gene loci towards tolerance to various problem soils and either modify or develop new cultivars for future need.

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Phenotyping Methods of Fungal Diseases, Parasitic Nematodes, and Weeds in Cool-Season Food Legumes

10

Seid Ahmed Kemal

Abstract

The productivity and production of crop plants are low in many parts of the world due to several biotic and abiotic stresses. The major biotic stresses are caused by foliar- and soil-borne diseases, parasitic weeds, and parasitic nematodes. Distribution and importance of diseases, parasitic weeds, and nematodes of cool-season food legumes are global and eco-regional in nature. For developing resistant germplasm, it requires systematic screening using field- and greenhouse-based techniques against the target biotic stresses. These phenotyping techniques have led to the identification of many varieties and germplasm currently in use by farmers and researchers. This chapter provides information on those techniques that are being employed to phenotype several diseases, parasitic nematodes, and weeds in cool-season food legumes.

Keywords

Food legumes • Foliar- and soil-borne diseases • Resistant • Screening methods

10.1 Introduction

Cool-season food legumes, viz., faba bean (*Vicia faba* L.), field pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), and grass pea (*Lathyrus sativus* L.), are important protein-rich crops. These crops are integral parts of rainfed agriculture and play a key role in the sustainability of cereal-based cropping systems. The present productivity of cool-season food legumes is constrained by several biotic and abiotic factors. The major biotic factors are fungal pathogens causing foliar and root diseases, as well as parasitic nematodes and weeds. Diseases of cool-season food legumes have either regional or global importance, and during epidemic periods, farmers can even lose their entire crops (Pande et al. 2009). Owing to the importance of diseases, one of the major components of disease management in many legume breeding programs is resistance breeding, and consequently, major achievements

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have been made in combating the ravages of key biotic constraints (Muehlbauer and Kaiser 1994; Tivoli et al. 2006; Muehlbauer and Chen 2007; Siddique et al. 2013). The identification of resistance gene pools is achieved through field and controlled screening techniques. Effective and efficient screening techniques are very critical in evaluating genetic materials (segregating populations, cultivated and wild germplasm accessions, and mapping populations).

Earlier screening techniques and their use in the identification of resistance gene pools from cultivated and wild genetic sources against fungal diseases, parasitic nematodes, and weeds have been reviewed by several workers (Nene et al. 1981; Cubero et al. 1994; Kraft et al. 1994; Muehlbauer and Kaiser 1994; Porto-Puglia et al. 1994; Sharma et al. 1994; Sillero et al. 2006; Infantino et al. 2006; Tivoli et al. 2006; Singh et al. 2007; Pande et al. 2011). This chapter has focused on various methods used to screen resistance to diseases, parasitic weeds and nematodes of cool-season food legumes, and attempts have been made to highlight key challenges and opportunities for phenotyping the diseases.

10.2 Phenotyping to Foliar Fungal Diseases

Cool-season food legumes are affected by many economically important foliar diseases caused by necrotrophic and biotrophic fungal pathogens. The major emphasis in the management of foliar diseases is mainly through resistance breeding that deploys resistance sources from cultivated and, to some extent, from wild relatives. The screening techniques are based on exposing the genetic resources of target crops to pathogen populations (single isolate or bulk populations) under field and controlled (greenhouse, plastic house, growth chamber, and laboratory) conditions. These screening techniques are discussed in the following text for the major foliar fungal diseases of important cool-season food legumes.

10.2.1 Chickpea

Chickpea is affected by foliar diseases like Ascochyta blight (*Didymella rabiei*), Botrytis gray mold (*Botrytis cinerea*), and rust (*Uromyces ciceri-arietini*). Ascochyta blight is a major production bottleneck almost in all chickpea-growing countries, while Botrytis gray mold (BGM) is a major problem in South Asia and Australia (Pande et al. 2006; Kaur et al. 2013). The National Agricultural Research Systems (NARS), International Center for Agricultural Research in the Dry Areas (ICARDA), and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) have put huge resources and time to develop cultivars resistant to key foliar diseases using genetic resources from cultivated and wild gene pools.

10.2.1.1 Ascochyta Blight

The field screening for Ascochyta blight is being done by spreading infected chickpea straws kept from previous seasons to initiate primary infections. If weather conditions (rainfall and temperature) are not conducive for Ascochyta blight development, spore suspensions of aggressive/virulent isolates are sprayed, and conducive conditions are created using sprinkler irrigation. At ICARDA, *Kabuli* chickpea Ascochyta blight nurseries (segregating populations, germplasm accession, and fixed lines) are screened each year at a large scale by planting susceptible genotypes along with the resistant genotypes at regular intervals. Inoculations are done by combining the spreading of infected chickpea straw at the seedling stage supplemented with artificial inoculation (two to three times) by spraying 10-day-old culture of the pathogen (10^5 spores/ml) using a tractor mounted sprayer, while knapsack sprayers are used for spraying the small plots. In countries where weather conditions are favorable (hot spot locations), disease nurseries are exposed to natural pathogen populations. The selected breeding lines from one season are usually evaluated in the subsequent season to avoid possible escapes before they are advanced for

yield trials following similar procedures described above.

Controlled screening techniques are used to confirm selections made from field screenings and study pathogen variability/virulence. For example, a highly virulent pathotype (pathotype IV) of *D. rabiei* is identified in few locations of Syria. This pathotype was used to expose all ICARDA *Kabuli* breeding lines and wild relatives under plastic house conditions in order to see its reactions on these genetic resources. Thus, such screening helps to avoid future threat to chickpea production from new emerging pathotypes (Imtiaz et al. 2011).

Different researchers use different seedling ages (12–15 days), ages of pathogen culture (7–12 days), temperature regimes (18–22 °C), and methods in creating relative humidity (RH) for seedling screening under controlled conditions. At ICARDA, breeding lines planted in pots (five seedlings of each entry/pot with a susceptible check in the center of each pot) are inoculated with 10-day-old culture of *D. rabiei* at 10^5 spores/ml. Inoculation is made until runoff and covered with plastic sheet for 72 h to create high relative humidity (RH). After inoculation, RH is kept above 80 % using humidifier or mist irrigation or spraying water by hand sprayers. The mini-dome seedling screening technique involves spraying of 2-week-old seedling with conidial suspension (2×10^5 spores/ml) to run off, and seedlings are immediately covered with an inverted translucent plastic cup in order to produce uniformly high relative humidity for 24 h to facilitate infections (Chen and Muehlbauer 2003; Chen et al. 2005). In cut-twig method, 80-day-old plant twigs with a minimum of five pinnules are collected, wrapped with a cotton plug, and placed in test tubes (15 × 100 mm) filled with sterilized distilled water. The twigs are transferred to a controlled environment facility, maintained at a temperature of 20 ± 1 °C, and a photoperiod of 12/12 h light regime. Inoculum is sprayed (5×10^4 spores/ml) on to the foliage using a hand-operated atomizer, and 100 % RH is provided for initial 4 days after inoculation, and later 100 % RH is maintained for 6–8 h for 10 days (Kottapalli et al. 2009; Pande et al. 2011).

10.2.1.2 Botrytis Gray Mold

In field screening, breeding lines are sown in 4-m-long rows (depending on seed availability) with susceptible cultivar acting as a spreader row after every two test entries. As flowering initiates, both test entries and infector-cum-indicator rows are sprayed with water through a sprinkler irrigation system to maintain high humidity to favor BGM development. To screen for BGM resistance in chickpea under growth room conditions, 10-day-old seedlings are grown in plastic trays and inoculated with conidial suspension of 3×10^5 spores/ml and incubated in growth room maintained at 15 ± 1 °C and 95–100 % RH with 12/12 h photoperiod regime (Sharma et al. 2013). In the cut-twig method, tender shoots of chickpea plants are cut in tray containing water, immediately wrapped in wet cotton plug, and placed into a test tube (15 × 100 mm) containing fresh tap water. Twigs are inoculated by spraying spore suspension of *B. cinerea* (10^4 spores /ml) and covered with moist polythene covers until symptoms develop (Kaur et al. 2013).

10.2.2 Faba Bean

Faba bean is affected by chocolate spot (*Botrytis fabae*), Ascochyta blight (*D. fabae*), rust (*Uromyces vicia-fabae*), and Cercospora leaf spot (*Cercospora zonata*).

10.2.2.1 Chocolate Spot

The field screening against the chocolate spot disease in faba bean requires humid conditions that favor disease epidemics. Such very high disease pressure development conditions commonly occur in coastal areas of Syria, Lebanon, and other hot spots locations in Morocco, Egypt, and Ethiopia. ICARDA faba bean breeding program uses these locations for evaluating its breeding materials and germplasm accessions

(Hanounik and Maliha 1986). When disease development is hampered by unfavorable weather conditions, artificial inoculations ($4-5 \times 10^5$ spores/ml) are done using mixtures of aggressive isolates, and high RH is created using sprinkler irrigation. In addition, spores of *B. fabae* can be mixed homogenously with sand, and infected straws can be applied one or more times at the vegetative stage of the crop (Villegas-Fernández et al. 2012).

Seedlings and detached leaves are used to screen faba bean germplasm accessions and breeding lines under controlled conditions. In the detached leaf technique, fully expanded leaflets of similar physiological age from the eighth node position are laid flat on a moistened filter paper kept on sterile benches. The cut end of each leaflet petiole is covered with moistened cotton to maintain leaves at maximum turgor. The upper side of the leaves is inoculated with one droplet on each half of each leaflet (5×10^5 spores/ml). Benches are covered with polythene sheets and incubated at room temperature (20 ± 2 °C) for 5–6 days. Petri dish (15×15 cm) containing water-agar (0.4 %) medium can also be used to screen genotypes using fully expanded leaves (Tivoli et al. 1986; Bouhassan et al. 2004).

10.2.2.2 Ascochyta Blight

Ascochyta blight development is initiated by spraying conidial suspension of *A. fabae* ($1–2 \times 10^5$ spores/ml) every 2 weeks and by spreading infested barley seeds (10 g/m^2) or infected faba bean seeds and infected straw immediately at planting or after seedling emergence (Maurin and Tivoli 1992; Sillero et al. 2001; Rubiales et al. 2012, 2013). Artificial inoculation is done using knapsack sprayer after sunset in order to take advantage of the darkness and high RH in the night to ensure high and uniform infections. Relative humidity can be increased through sprinkler irrigation several times a day. Susceptible and resistant check entries are planted after every two to six test entries.

Under controlled conditions, seedlings are inoculated by spraying spore suspension (5×10^5 spores/ml) and incubated for 24–48 h in darkness with 100 % RH and kept at 20 °C with a 12–14/10–12 h photoperiod regime in a growth chamber (Hanounik and Robertson 1989; Avila et al. 2004). In a detached leaf technique, young leaves (one to three from the top of the plant) are inoculated with a spore suspension (5×10^4 spores/ml) and maintained in good conditions for more than 10 days for symptoms development and disease scoring (Kohpina et al. 2000).

10.2.2.3 Cercospora Leaf Spot

Phenotyping methods for screening against Cercospora leaf blight disease under field and controlled conditions have been developed in Australia (Kimber and Paull 2011). Natural disease epidemics developed from primary inoculum existing under field condition from straws left from previous seasons are used to screen faba bean genetic resources. For screening under controlled conditions, spores and/or mycelia fragments (10^5 spores or fragments/ml) from different isolates are mixed, and faba bean seedlings at the four-leaf nodes stage are inoculated until runoff. High RH is maintained using overhead ultrafine misting for 30 s every 2 h and kept at 18 °C (Kimber and Paull 2011).

10.2.3 Lentil

Lentil is affected by Ascochyta blight (*D. lentis*), BGM (*B. cinerea*), anthracnose (*Colletotrichum truncatum*), rust (*U. vicia-fabae*), Stemphylium blight (*Stemphylium botryosum*), and downy mildew (*Peronospora viciae*). Various approaches are used to phenotype germplasm against these diseases in order to identify resistant donors for improving the host plant resistance in lentil.

10.2.3.1 Ascochyta Blight

Ascochyta blight screening nurseries are planted in hot spot areas where environmental conditions are conducive and inoculum sources are present for disease development. Disease development can be further increased using spreader rows throughout disease nurseries that act as the secondary source of inoculum. Moreover, infected straws and infested lentil seeds as well as spore

suspension can be sprayed in the disease screening fields to initiate disease foci (Ahmed and Morrall 1996), and stubble may be spread over the test site to initiate disease epidemics. Different researchers used different spore loads of *D. lentis* ranging from 5×10^4 (Singh et al. 1982) to $2–5 \times 10^5$ spores/ml (Ahmed and Morrall 1996; Tar'an et al. 2003) to inoculate lentil seedlings under controlled conditions. Ten-day-old seedlings can be inoculated with 2×10^5 spores/ml until runoff can be incubated for 48 h under a growth chamber at 20 °C under 100 % RH.

10.2.3.2 Anthracnose

Field screening for anthracnose resistance depends on use of spore suspensions, spreading infected residue from the previous cropping season and sterilized wheat grains (10 g m^2 every 2 weeks) colonized with the pathogen (Buchwaldt et al. 2003). For field inoculation of lentil at the late vegetative or early flowering stage, with spore suspension (4×10^4 spores/ml), plots are first saturated with water to increase relative humidity. Inoculated plots are covered with polyethylene sheets overnight to maintain high relative humidity (Buchwaldt et al. 2003).

For greenhouse screenings, breeding lines along with susceptible and resistant checks grown in pots (six to eight seeds/pot) are inoculated with single isolate spore suspensions (10^5spores/ml) at 10th–12th node stage or early flowering stage and incubated for 24 h at 100 % RH (Buchwaldt et al. 2003; Tar'an et al. 2003; Tullu et al. 2003). After incubation, seedlings are transferred to a greenhouse maintained at 20–24/15–19 °C day/night temperature regime (Buchwaldt et al. 2013; Shaikh et al. 2013).

10.2.3.3 Botrytis Gray Mold

Screening for BGM resistance is conducted in field nurseries inoculated with spore suspensions (3×10^5 spores/ml) or by spreading infected stubble or planting nurseries after faba bean or lentil crop to get natural inoculum (Davidson et al. 2004; Lindbeck et al. 2008). To favor disease development, high seeding rate (Lindbeck et al. 2008) and sprinkler irrigation are recommended.

For screening under controlled conditions, high RH and temperatures at least 20 °C and inoculation with spore suspension (10^4 spores/ml) are optimal conditions for good disease development. Initiation of disease development after disease infection is greatly aggravated by the presence of senescent leaves within 2 days from the inoculation to sporulation on the test entries and checks.

10.2.3.4 Stemphylium Blight

The prevalence of moderate to warm temperatures (25–30 °C) and high RH favor disease development. These conditions prevail naturally in Northeastern India and west-central Bangladesh and, hence, suitable for field screening against Stemphylium blight. Currently, field screenings are mainly done in Canada (Saskatchewan) and Bangladesh where disease development is very high during most cropping seasons (Podder et al. 2013). For screening under controlled conditions, optimum sporulation is initiated by incubating the pathogen for 7–10 days at 27 °C under cool fluorescent light and 16/8 h light regime. Three-week-old whole lentil plants can be inoculated at 25–30 °C with a leaf wetness period of 48 h (Mwakutuya and Banniza 2010).

10.2.4 Field Pea

The major foliar diseases of field pea are Ascochyta blight complex (*Didymella* spp. and *Phoma medicaginis* var. *pinodella*), powdery mildew (*Erysiphe pisi*), downy mildew (*Peronospora viciae* f. sp. *pisi*), and rust (*U. vicia-fabae* and *U. pisi*). Ascochyta blight is the most common disease in field pea in many countries and is caused by a complex of pathogens. *Didymella pinodes* and *D. pisi* are considered to be the primary pathogens in Ascochyta blight disease complex (Davidson et al. 2011).

10.2.4.1 Ascochyta Blight Complex

Many researchers use infected pea stubble, spreading of barley or sugar beet grains infected with various isolates or allow a natural epidemic in hot spot areas for field screening against Ascochyta blights in field pea. Alternatively, spore suspensions of mixed isolates collected from different areas are sprayed for disease development.

Detached leaflets and stipules screening techniques are mostly used for phenotyping under the controlled conditions. Heath and Wood (1969) proposed such method of screening where short stem segments with attached stipules from third to fourth nodes are floated lower surface down on tap water in a compartmented square Petri dish (12 cm). Inoculation with a drop of 10 ml of spore suspension is done on the upper surface of the stipules, avoiding the main veins. To avoid drop evaporation, Petri dishes are placed into large transparent plastic boxes. Inoculated-detached stipules are incubated in a growth chamber for an initial period of 18 h in the dark followed by 7 days with 14/10 h light regime at 20 °C (Onfroy et al. 2007).

10.3 Phenotyping for Other Diseases of Cool-Season Food Legumes

10.3.1 Rust (*Uromyces* spp.)

Rust can cause up to 100 % yield loss on lentil when environmental conditions are conducive for disease development. *Uromyces vicia-fabae* affects lentil, faba bean, grass pea, and field pea. Field pea is also affected by *U. pisi* (a heteroecious pathogen), while *U. ciceri-arietini* affects chickpea. Rusts can be one of the threats of food legumes under climate change in many countries where the diseases are reported to be sporadic. Hence, there is a need to do anticipatory chickpea breeding for rust resistance in some countries. The best way to phenotype against rust (*U. vicia-fabae*) in lentil, faba bean, and field pea is to test the germplasm in hot spot areas. Many locations in Morocco, Ethiopia, India, Bangladesh, and Egypt have been identified as hot spots to screen for rust resistance. In India, field pea rust screening is done by planting germplasm in November to favor disease development, and plants are maintained in the vegetative phase by bud clipping. Further, spreader rows are planted after every ten test entries as well as around the disease plots for the epidemic development of disease. Spore suspension (10^4 spores/ml) can be applied to supplement natural inoculum and later irrigate the field to maintain adequate humidity for rust development (Chand et al. 2006; Rai et al. 2011). In Spain, field pea breeding lines are planted in hot spot areas for *U. pisi* infection and disease development and augmented by spraying an aqueous spore suspension (6×10^4 spores/ml) from a bulk pathogen population collected from different areas (Barilli et al. 2009a, b). Sprinkler irrigation can be used to increase RH to create a more favorable environment for rust infection.

Similarly, rust screening in chickpea is also done in hot spot areas. For example, in Ethiopia, chickpea rust appears during rainy season (February–April), and, therefore, field screening for rust can be done during this period under natural infection. In order to augment natural disease development, disease nurseries can be inoculated by spraying with an aqueous spore suspension (200 mg spores/l) after sunset to benefit from the darkness and high RH at night. For screening against chickpea rust in growth chamber, seedlings at the fourth leaf stage are inoculated by dusting freshly collected rust spores (2 mg spores/plant) diluted by mixing with pure talc of single spore or bulk population of the pathogen and incubated for 24 h at 20 °C in complete darkness at 100 % RH. After this, seedlings are moved to a growth chamber at 20 °C with a 13–14/10–11 h light regime (Sillero et al. 2012). Stuteville et al. (2010) inoculated seedlings of chickpea and other legume species by spraying freshly collected urediniospores of *U. ciceri-arietini* at a rate of 100 mg/100 ml distilled water containing two drops of Tween 20 until runoff and immediately enclosed

seedlings in plastic boxes to maintain near 100 % RH. The boxes are placed in darkness at 20 °C in a growth chamber for 24 h and incubated growth chamber or plastic house with 16/14 h light regime at 25 °C. In field pea, seedlings (three to four fully expanded leaves stages) are inoculated by dusting freshly collected urediniospores (0.5–2 mg spores/plant) mixed with pure talcum powder using single spore or bulk spores of different isolates. Inoculated seedlings were incubated at 100 % RH at 20 °C in darkness for 24 h under 14/10 h light regime (Emeran et al. 2005). Herath et al. (2001) proposed detached leaf technique for screening rust resistance genotypes in faba bean. In this technique, detached leaflets are carefully laid, adaxial surface up, in Petri dishes with moistened tissue paper with distilled water containing benzimidazole (1 %) and then inoculated with spore suspension.

10.3.2 Powdery Mildew (*Erysiphe pisi*)

This is one of the most serious diseases of field pea. Screening of germplasm of cool-season food legumes is taken up in fields where powdery mildew is a recurrent problem. Infector rows of susceptible checks are planted to ensure uniform spread of the disease. To augment natural disease development, artificial inoculation is recommended by tapping conidia from heavily infected plant parts. Genetic resources showing resistance for two seasons in field screening can be retested under artificial conditions against prevalent pathogen populations. In Ethiopia and India, field pea screening against powdery mildew is done under natural epidemics (Fikere et al. 2010; Rana et al. 2013). Similar screening is also done for this disease in grass pea. However, highly susceptible field pea varieties are used around the screening plots in order to spread the disease (Vaz Patto et al. 2006).

Under controlled conditions, seedlings and detached leaves are used by many researchers to identify powdery mildew resistance in field pea and grass pea. In grass pea, seedlings are evaluated in growth chamber where disease spreads from inoculated susceptible field pea cultivars planted around the pots. In detached leaf technique, cut leaves are placed in Petri dishes on filter paper or sheets of cotton, containing a solution of 5 % of sucrose or floating them on this solution or on tap water (Smith et al. 1996; Banyal and Tyagi 1997; Viljanen-Rollinson et al. 1998; Rana et al. 2013).

10.3.3 Downy Mildew

Several methods have been developed to phenotype field pea germplasm against downy mildew under controlled conditions (Ryan 1971; Stegmark 1991). Soil infested with oospores is mixed in field above the seeds. Germinated seeds are soaked in a conidial mixture, and conidial spores are also sprayed onto seedlings. In order to induce sporulation, plants are placed in high RH (100 %), either by covering with polyethylene sheets and misting with water or by placing plants in a moist chamber at 12–15 °C for 24 h. Automization of conidial suspension (2×10^4 to 5×10^5spores/ml) on 3–4-week-old field pea seedlings is generally preferred (Thomas and Kenyon 2004). After inoculation, seedlings are kept under high RH for 24–48 h and then at 16–20 °C for 7–20 days, after which high RH is again maintained to induce sporulation (Gill and Davidson 2005). Systemic downy mildew infection is developed by inoculating 7-day-old seedlings with four droplets (1×10^6 spores/ml) of conidial mixture of pathogen on the apical buds. These seedlings are then incubated for 4 days at 4 °C in high RH and after this, kept for 17 days at 12 °C/4 °C day/night temperature cycles. Subsequently, low temperature and high humidity are reapplied for 48 h in order to stimulate sporulation (Davidson et al. 2004). In case of faba bean, seedlings (four to five leaf stages) are inoculated with *P. viciae* conidial suspension (1×10^4 spores/ml). These inoculated seedlings are enclosed in black polythene bags for 48 h at 10 °C after inoculation. After this, the seedlings are reopened from bags, but temperature is still maintained at 10 °C for 14–18 days and enclosed in bags again for further 48 h in order to promote

sporulation (Thomas and Camps 1997). In Syria, high levels of down mildew infection occur among the genotypes planted early (November) under zero tillage conditions. These conditions can be used for phenotyping lentil genotypes against downy mildew disease in the future (Ahmed and Morrall 2012).

10.4 Screening Techniques for Soil-Borne Fungal Diseases

Many soil-borne pathogenic fungi causing wilt/root rot complex affect the cool-season food legumes leading to seed rotting as well as seedling and adult plant mortality. Therefore, both field and artificial screening techniques have been developed against major soil-borne diseases, especially wilt and root rots.

10.4.1 Fusarium Wilts

Field screening against Fusarium wilt is taken up in sick plots developed by repeated planting of susceptible genotypes (Nene et al. 1981; Kraft et al. 1994; Infantino et al. 2006). Wilt-sick plots for chickpea have been developed and widely used in different international institutes (ICARDA, ICRISAT) and countries including India, Tunisia, Sudan, Ethiopia, and Spain. Chickpea germplasm is planted in 2–4 m rows (20–40 seeds/row) with susceptible genotypes planted after every two to four test entries in two replications. The putative wilt resistant germplasm selected in the first year are reevaluated in the second season to confirm their resistance. For lentil, Fusarium wilt (*F. oxysporum* f. sp. *lentis*) sick plots have been developed at ICARDA, India, Ethiopia, and Sudan. Lentil test entries are planted in rows of 2–4 m length (25–50 seeds/row) alternating with appropriate susceptible checks after every two to four test entries at ICARDA. It is important to consider sowing a highly resistant line after every tenth row (Bayaa et al. 1997). For field pea, Fusarium wilt (*F. oxysporum* f. sp. *pisi*) sick plot is established at Washington State University, Spillman Research Farm near Pullman, WA, by depositing infested soil with the pathogen, and genetic materials are sown in single rows, 1.5 m long with a susceptible check at regular intervals to monitor disease uniformity. For artificial screening against Fusarium wilt, chickpea and lentil test entries are planted in pots filled with infested soil with target pathogens, or roots of seedlings grown on sand are dipped in known spore suspension and planted in pots filled with sterilized soil (Nene and Haware 1980; Kraft et al. 1994; Sharma et al. 2005; Infantino et al. 2006). In field pea, 7-day-old seedlings are inoculated after roots are trimmed and immersed for 5 min in a spore suspension (5×10^6 spores/ml) of *F. oxysporum* f. sp. *pisi* and incubated in a controlled environmental condition (Bani et al. 2012) under a 16/8 h light regime at 26 ± 2 °C. Selected breeding materials are reevaluated following the same procedure described above.

10.4.2 Root Rots

Root rots of cool-season food legumes are caused by many pathogenic fungi, and only selected screening techniques for those diseases where host plant resistance have been found useful are reviewed. Sick plots to screen faba bean against Fusarium root rot (*F. solani*) have been developed in Ethiopia and Sudan where the disease is very important under waterlogged conditions in black soil. Breeding lines and germplasm accessions are planted with susceptible checks after every two to four test entries. Field screening of chickpea for Phytophthora root rot resistance (*Phytophthora megasperma* f. sp. *medicaginis* (*Pmm*)) is done in highly infested plots, and in some conditions, disease pressure is increased by inoculating aggressive mixtures of *Pmm* isolates collected from different areas (Brinsmead et al. 1985; Du et al. 2013). Aphanomyces root rot sick plot is developed to increase the level of resistance in field pea in the USA, France, and New Zealand. In Canada, field pea screening against Fusarium root rot (*F. solani* f. sp. *pisi*) is done by multiplying the

pathogen on sterilized wheat grains at 25 °C for 3 weeks, drying and mixing 10 g of inoculum with 15 seeds of test genotypes, and planting in a single row of 1 m length in the field (Feng et al. 2011).

Under controlled conditions, chickpea genetic resources can be screened against dry root rot (*Rhizoctonia bataticola*) by growing test entries with susceptible check for 7 days in sterilized sand. After this, seedlings are uprooted and inoculated by macerated fungal mass for 1 min. Inoculated seedlings are placed in folded, moist blotting paper with the shoots left outside, then incubated at 35 °C with 12/12 h photoperiod (Pande et al. 2006). For Phytophthora root rot, chickpea seedlings are grown in a glasshouse under 27/23 °C day/night temperature at 12/12 h light regime and flooded by keeping the pots inside watertight pots filled to the level of the soil surface with water at 17 days of sowing (Du et al. 2013). Seedlings are inoculated with 40 ml of zoospore of *Pmm* suspension produced from 10-day-old cultures poured evenly around the base of the plants, and saturated soil conditions are maintained for 7 days.

10.5 Phenotyping for Parasitic Weeds

Cool-season food legumes suffer from parasitic weeds (*Orobanche* and *Phelipanche* spp.) in the Mediterranean regions. As a result, faba bean acreage and production have declined over years, and now these weeds are spreading in Ethiopia and Sudan. Field dodder (*Cuscuta* spp.) is also reported on chickpea in Syria, Israel, the USA, and Uzbekistan. Efforts have been made to develop resistant/tolerant varieties mainly in faba bean to these parasitic weeds. Uniform distribution of the parasite weed seeds in the soil in order to prevent escapes is required for phenotyping against these weeds in field. Thus, highly infested plots are used to select potentially resistant genotypes. For small-scale screening, plots can be artificially infested by mixing parasitic weed seeds with sand and applying them along the rows at sowing time. It is recommended that each test entry should be surrounded by rows of a susceptible check as a reference. This has been successfully applied in faba bean breeding program at ICARDA using a highly infested plot with *O. crenata* (Maalouf et al. 2011). Similar technique has also been followed recently in lentil for screening wild relatives against parasitic weeds. Field screenings reported on chickpea using highly infested fields have been successfully used to identify some resistance source against field dodder (Goldwasser et al. 2012).

Several methodologies (substrate, pot size, etc.) are developed and used to screen cool-season food legumes for parasitic weed resistance under controlled conditions. The phenotyping against such parasitic weeds requires substrates that allow good plant growth and broomrape infection. These substrates can be easily washed from the roots and facilitate observation of parasite development. Linke et al. (1991) suggested the use of pots mixed with 7,500 (about 30 mg) seeds/kg substrate to screen chickpea for broomrape resistance. The use of Petri dishes is suggested by Sauerborn et al. (1987) for mass screenings of grain and model legumes under controlled conditions. Subsequently, many researchers used this method with modifications (Sillero et al. 2001; Rodríguez-Conde et al. 2004; Pérez-de-Luque et al. 2005). The mini-rhizotron technique can be used to screen a small number of breeding lines against *Orobanche* and *Phelipanche* spp. (Fernández-Aparicio et al. 2012).

For screening of faba bean using the mini-rhizotron, seeds of parasitic weeds are surface sterilized with formaldehyde 0.2 % and 0.02 % of Tween 20, rinsed thoroughly with sterile distilled water and dried for 1 h in a laminar air flow over glass-fiber filter paper (GFFP, Whatman GF/A) at a density of 50 seeds/cm^2. Thirty GFFPs are individually placed over a square Petri dish filled with sterile perlite moistened with sterile distilled water. Petri dishes are placed in the dark at 20 °C for 11 days. Susceptible faba bean genotype is surface sterilized with 2 % sodium hypochlorite solution for 5 min and then rinsed thoroughly with sterile distilled water

and germinated in Petri dishes with moistened filter papers placed for 4 days in a growth chamber under dark warm (20 °C) conditions. Seedlings are transferred individually to GFFP sheets and placed into the Petri dishes, punctured on the top so that plant foliage can develop outside of the dish. Petri dishes are sealed with parafilm, wrapped in aluminum foil, and stored vertically in a growth chamber (20 °C and 12/12 h light regime). Seedlings are supplied with modified Hoagland's nutrient solution at one-quarter strength (30 ml/dish) twice per week (Fernández-Aparicio et al. 2012). After 7 days, the GFFP is replaced by another GFFP containing the conditioned parasitic seeds allowing the intimate contact of parasitic seeds with the root exudates.

Chickpea screening for dodder resistance under controlled condition is done by removing seeds from matured dry field dodder plants, air-dried, threshed, cleaned and stored at 4 °C until use. Before sowing, dodder seeds are scarified by soaking in sulfuric acid for 1 h; washed and air -dried. Chickpea seeds are sown in 15-cm-diameter pots and placed in a heated glasshouse (10 °C min and 30 °C max temperatures). Inoculation of dodder seeds is performed by placing about 50 seeds around each chickpea plant stem at 0.5 cm depth (Goldwasser et al. 2012).

10.6 Parasitic Nematodes

Parasitic nematodes (*Heterodera*, *Pratylenchus*, *Meloidogyne*, and *Ditylenchus* spp.) are key production problems in cool-season food legumes in different countries (Sharma et al. 1994; Castillo et al. 2008). Field screening against parasitic nematodes is done using sick plots. For example, chickpea germplasm is screened for cyst nematode (*H. ciceri*) resistance in sick plot developed at ICARDA, Syria. Faba bean screening against stem nematode (*D. dipsaci*) is done by mixing infected stems with soil, and after 2 weeks, the infested soil is diluted with a nematode-free soil until a population density of about 300 larvae per 1,000 cm^3 soil is obtained. Seeds are sown in rows 1 m long and 50 cm apart. A susceptible cultivar row is planted after every five test rows. All seeds are covered with infested soil to a depth of 15 cm. Screening chickpea germplasm against root-knot nematodes (*Meloidogyne* spp.) is done on naturally infested fields which is enhanced by growing susceptible cultivars, and then test entries are sown in 2–4 m rows together with highly susceptible genotypes after every ten test entries (Sharma et al. 1994). To screen chickpea genetic materials for resistance to root-lesion nematodes, planting is done in highly infested soil with *Pratylenchus* spp. and/or can be augmented with artificial inoculations (Thompson et al. 2011).

In controlled screening for cyst nematodes (*H. ciceri*), the nematodes are reared in susceptible chickpea genotype under greenhouse condition, extracted, dried under shade, and mixed with sterilized sand to give 4,000–6,000 eggs/g of soil (Di Vito et al. 1996). The test entries are planted in pots (four plants/pot) filled with steam-sterilized soil artificially infested with 20 or more eggs and juveniles/g and incubated in a greenhouse at 20 ± 5 °C. For stem nematode screening, inoculum is multiplied on susceptible faba bean cultivars or on callus tissue and extracted with the incubation or centrifuge methods. Five-day-old faba bean seedlings are transplanted into glass tubes or in small pots filled with organic sterilized compost, and after a week, seedlings are inoculated with the nematode suspension in the leaf axil and incubated at 15–20 °C in a growth chamber. High RH is required for several days to favor nematode infection. For root-knot nematodes, chickpea seedlings are planted in infested soil in pots, and 5–10 day old seedlings are inoculated with 5,000–10,000 eggs/plant by pouring the egg suspension into four (3–5-cm-deep) holes around the stem base. After inoculation, pots are irrigated lightly to assure survival and even distribution of the nematode inoculum and kept at 25 ± 5 °C in a greenhouse (Sharma et al. 1994).

10.7 Multilocation Testing for Resistance

The evaluation of genetic resources in multilocation testing is a common phenotyping strategy in almost all national and international breeding programs to expose breeding materials to wide ranges of pathogen and parasitic weed populations. ICARDA evaluates its germplasm in different regions through its International Disease and Parasitic Weed Nurseries platform. These nurseries are sent to hot spot areas where diseases and parasitic weeds are the key production constraints appearing every cropping seasons in epidemic proportions. Recent multilocation testing of faba bean and chickpea against Ascochyta blights (Rubiales et al. 2012; Pande et al. 2013), chickpea against BGM (Sharma et al. 2013), and faba bean against parasitic weeds (Maalouf et al. 2011) were able to identify genetic resources with good levels of resistance across locations.

10.8 Challenges and Opportunities for Phenotyping to Resistance Breeding

Conventional phenotyping techniques will continue to play a vital role in developing germplasm pools resistant to key biotic constraints. Nevertheless, there are several limitations of using conventional phenotyping techniques against diseases under field and controlled conditions. Firstly, field screening techniques require time and space. The variations in pathogen populations lead to variable results. Environmental conditions and disease measurements also affect the observations taken on diseases. Secondly, screening under controlled conditions is not standardized, and replications across different labs do not always give similar results. Thirdly, resistance against most diseases is quantitatively inherited, and hence, phenotyping is a big challenge both under field and controlled conditions. Fourthly, assessments of diseases, nematodes, and parasitic weeds are largely based on visual scoring that consumes time and can generate bias results among different raters and experimental repeats (Poland and Nelson 2011). Finally, weak correlations of seedling and adult plant resistance as well as different disease measurements do not always give acceptable results. Moreover, phenotyping for multiple resistances is still a daunting task for breeders and plant pathologists (Nene 1988). In most cases, screening for multiple resistances is done by exposing similar genetic resources for individual diseases. In soil-borne diseases, some achievements have been made for wilt/roots complex in chickpea and lentil.

The constraints of field phenotyping have driven intense interest over the past decades to overcome the above key challenges. Therefore, the evolution of molecular technologies (Torres et al. 2006; Gaur et al. 2012; Kumar et al. 2012; Varshney et al. 2012; see Chap. 18) and remote sensing technologies (see Chap. 14) has opened new opportunities for phenotyping against the diseases. Compared to genotyping, modern phenotyping technologies (Scholes and Rolfe 2009; White et al. 2012) have limited applications in plant resistance breeding. In this scenario, the modern phenotyping techniques based on imaging which have been discussed in details in Chap. 5 can play a key role to identify quantitatively inherited traits (Rousseau et al. 2013). The use of new phenotyping methods has been successfully applied in sugar beet to identify resistant lines against Cercospora leaf spot (Chaerle et al. 2007), Fusarium wilt in cotton, and aphid resistance in *Arabidopsis thaliana* (Chen et al. 2011). The field-based phenotyping platforms for some host-pathogen systems and phenomic projects (Furbank 2009; Furbank and Tester 2012; Houle et al. 2010) will help in the future for phenotyping against diseases.

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Advances in Phenotyping of Functional Traits

11

Charles Y. Chen, Christopher L. Butts, Phat M. Dang, and Ming Li Wang

Abstract

Phenotyping is analyzing a plant's phenotype and providing a critical means to understand morphological, biochemical, and physiological principles in the control of basic plant functions as well as to select superior genotypes in plant breeding. Besides well-known classical plant phenotyping procedures based on visual observations, measurements, or biochemical analyses, many recent developments are target specific and highly automated analysis procedures. Automated phenotyping approaches are far more successful at the laboratory and greenhouse scale than in field conditions where many other variable factors complicate the retrieval of imaging data collected in the field. With respect to plant breeding, rapid measurement procedures, a high throughput, a high degree of automation, and an access to appropriate, well-conceived databases are required to depict the performance of certain genotypes in the field. This chapter will focus on destructive, nondestructive, and automated techniques available to quantify plant morphological and biomass traits, root system architecture, physiological functional traits, biochemical quality and nutritional compositions, and postharvest characteristics.

11.1 Introduction

In plants, functional traits are morphological, biochemical, physiological, structural, phenological, or behavioral characteristics that are expressed in phenotypes of individual plants, which have relevance to the plant's function playing ecosystem roles or affect their performance. By themselves, functional traits govern the organism's effects on ecosystem processes referring to effect traits and/or its response to

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the physical and biotic environment pressures called response traits (Violle et al. 2007). Plant phenotyping attempts to quantify functional traits that involve plant quality, photosynthesis, development, architecture, growth, and biomass productivity of single plants using different analytical procedures. Phenotyping provides a critical means to understand morphological, biochemical, and physiological principles in the control of basic plant functions as well as to select superior genotypes in plant breeding. Besides well-known classical plant phenotyping procedures based on visual observations, measurements, or biochemical analyses, many recently developed analytical methods are target specific and are highly automated. The technological developments for laboratory or greenhouse-based phenotyping have been dramatically improved, complemented by other techniques, and brought to a platform of high throughput. Automated phenotyping approaches are far more successful at the laboratory and greenhouse scale than in field conditions where many other variable factors complicate the retrieval of imaging data collected in the field. With respect to plant breeding, rapid measurement procedures, a high throughput, a high degree of automation, and an access to appropriate, well-conceived databases are required to depict the performance of certain genotypes in the field. This chapter will focus on destructive, nondestructive, and automated techniques available to quantify plant morphological and biomass traits, root system architecture, physiological functional traits, biochemical quality and nutritional compositions, and postharvest characteristics.

11.2 Plant Morphology and Biomass Traits

Phenotypic evaluation of crop genotypes such as breeding lines, germplasm accessions, and mapping populations is a fundamentally important procedure for plant breeding and genetic research. The widest application of advanced phenotyping is to determine morphological characters such as plant height, total leaf area, leaf number, or canopy width and shape from images taken of individual plants using platforms that combine robotics and image analysis with controlled environment systems (Arvidsson et al. 2011) or with field-based phenotyping (FBP) (White et al. 2012).

The use of greenhouses and controlled environments to represent field environments for certain targeted applications has proven beneficial in rice research (Reuzeau et al. 2005; De Wolf et al. 2008). Automated greenhouses, in which plants are grown and analyzed automatically using images taken at regular intervals, are the basis for high-throughput phenotyping. In these approaches, single plants are usually analyzed in a static context, in which a range of plant genotypes are exposed in a given set of environmental conditions such as water availability, continuous lighting, and temperature conditions. Various fully automatic high-throughput plant growth and phenotyping platforms have been developed. The French National Institute for Agricultural Research (INRA) uses an automated platform (PHENOPSIS; Optimalog, Saint-Cyr-sur-Loire, France) to phenotype plant responses to soil water deficit in *Arabidopsis thaliana* for the identification of an accession with low sensitivity to soil water deficit (Granier et al. 2006). Similar to PHENOPSIS, an in-house system, GROWSCREEN, was designed for rapid optical phenotyping of different plant species by quantifying the dynamics of seedling growth acclimation in response to altered light conditions. The Research Center Jülich adopted GROWSCREEN to analyze phenotypes of different plant species (Walter et al. 2007). Both PHENOPSIS and GROWSCREEN use a camera which is moved over the plants. The high-throughput platform that enables large-scale phenotyping of plants in a fully automatic fashion (TraitMill™; CropDesign, Zwijnaarde, Belgium) has been used to identify yield-enhancement genes for the transgenesis and plant evaluation in rice (*Oryza sativa* L.) (Reuzeau et al. 2005). Quantitative, nondestructive analysis systems have been developed using

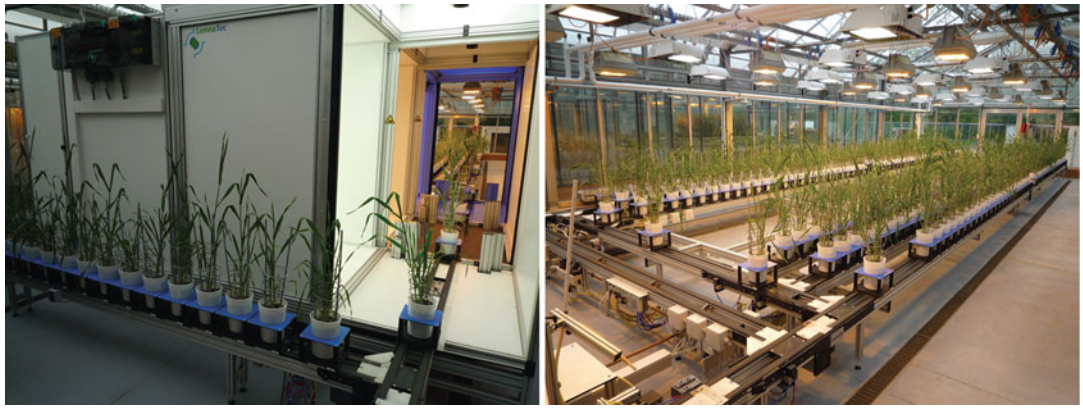

Fig. 11.1 View of Scanalyzer 3D platform. Image acquisition device for images in visible, near-infrared, and ultraviolet spectra (*left*), greenhouse device consisting of a conveyor belt system carrying 600 plants (*right*) (LemnaTec 2013)

multiple three-dimensional camera units to record multispectral images of plants to provide a diversity of phenotyping information for multiple crops or model plant systems in fully automated greenhouses (Scanalyzer 3D; LemnaTec GmbH, Aachen, Germany). The system captures images in wavelengths from far infrared to ultraviolet (Fig. 11.1). This system is used in several laboratories around the world, such as the Australian Centre for Plant Functional Genomics (ACPFG) in Adelaide, Australia; the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben; Agrobios, Italy; KeyGene, the Netherlands; Bayer CropScience, Belgium; DuPont Pioneer, USA; and BASF, USA. The system called "The Plant Accelerator" is a facility for Australian plant scientists, built around the LemnaTec (2013) Scanalyzer 3D platform in ACPFG in Australia. It consists of four Smarthouses™, fully climate-controlled greenhouses equipped with computer-controlled conveyor belts carrying up to 600 plants per room. Plants are grown in individual carts situated on a conveyor system. Each carrier is labeled with a radio frequency identification (RFID) for full traceability of that plant and the corresponding data throughout the course of an experiment. RFID can be used to control watering and nutrient supplementation for single plants. Besides managing plant movement and tracking, the conveyor system can automatically rotate plant locations throughout an experiment to reduce possible positional effects. Each Smarthouse is connected to one of the two imaging cabinets. There are five imaging chambers within each of the two imaging cabinets. Cameras in these chambers collect top and two orthogonal side view images of plants at multiple wavelengths and modes, including far infrared and visible reflectance and UV fluorescence, providing a diversity of phenotype information. Cameras capture images in the visible spectrum to quantify the overall plant skeleton analysis which is the key to growth dynamics, morphology, and architecture such as separation of stem and leaves, information about nodes, length of leaves, plant color classification—key to plant health—stress, nutrients and senescence, shoot mass, and other physical characteristics. Near-infrared images are used to measure water content, distribution, and dynamics in the leaves and soil. Far-infrared imaging provides information about leaf temperature and transpiration rate, while UV lighting detects chlorophyll and green fluorescent protein (GFP) fluorescence. The throughput of each of these imaging halls is sufficient to record data from all the plants in one Smarthouse in a single day. An extensive amount of data is generated by the platform, and the total capacity in the accelerator is up to 2,400 plants that can be phenotyped three times a week. The data collected from the accelerator is stored in a

database system for analysis by Lemna Launcher managing all software processes.

The Plant Accelerator offers many benefits. Among them, high throughput is considered the most significant. With the capacity to record dozens of parameters on up to 1,200 plants per day, it enables the identification of rare events that can be studied without automation technology. Since measurements are taken in full objective and under very controlled conditions, a high degree of accuracy and reproducibility can be achieved with the system.

Limited greenhouse space or chamber volumes often inhibit the ability to allow plants to flower and set seed due to insufficient soil volume to supply adequate nutrients and water for normal plant growth, making it impossible to assess normal patterns of growth. Field-based phenotyping (FBP) has been gradually accepted as an appropriate approach to delivering the requisite throughput in terms of numbers of plants or populations, as well as an accurate description of trait expression in the real-world farming environment. A recently published paper by White et al. (2012) comprehensively reviewed field-based phenomics for plant genetic research, in which key criteria, experimental approaches, and equipment and data analysis tools required for robust, high-throughput field-based phenotyping (FBP) were described. White et al. (2012) pointed out that an FBP platform requires six components: "(1) instruments for acquiring raw data from field plots; (2) physical systems for integrating different instruments including providing power, data logging or transmission, partial or complete shading, and protection from dust, vibration, and adverse weather; (3) vehicles for positioning the instrument rapidly and accurately in a field; (4) high-throughput analytic capabilities to complement field measurements (e.g., of leaf or seed samples); (5) software systems for managing and analyzing potentially large and complex datasets; and (6) integrated management protocols to maximize reliability and efficiency of the phenotyping." The technologies that enable FBP to characterize multiple targeted traits include photodiodes, high-intensity light-emitting diodes (Yeh and Chung 2009), infrared imagery using digital cameras and accurate infrared thermometers (IRT, French et al. 2007), stereo image analysis (Biskup et al. 2007; Yu et al. 2007), acoustic-based distance sensing (Ruixiu et al. 1989; Andrade-Sanchez et al. 2012), chlorophyll fluorescence meter (Kolber et al. 2005), and laser distance sensing and near-infrared spectroscopy. In order to rapidly and accurately position instruments over field plots, or even individual plants, several vehicle options used in FBP include high-clearance tractors (Schleicher et al. 2003; Andrade-Sanchez et al. 2012), linear move or central pivot irrigation systems (Kostrzewski et al. 2003; Colaizzi et al. 2003; Haberland et al. 2010), manned fixed- and rotary-wing aircraft (French et al. 2007), unmanned aircraft (Hunt et al. 2005; Hakala et al. 2010), and tethered aerostats (Jensen et al. 2007; Ritchie et al. 2008). Each option has its strengths and weaknesses, and the selected option should complement the research objectives. For instance, studies that require continuous measurements over 24 h or longer periods might use cable-suspended robots, for example, those established at the National Institute of Standards and Technology (NIST) in the 1980s (Albus et al. 1993), to provide an option for a vehicle that can operate continuously over a field but in a limited area. However, manned helicopters represent a mature technology capable of carrying a large payload and supplying power to an instrument system, but cannot be used in close proximity to plants due to rotor downwash and country-specific administration regulations restricting the minimum flying altitude. Thus, only imaging is appropriate to resolve plots. Unmanned helicopters are a promising alternative to manned aircraft since they allow flying at much lower altitudes (Berni et al. 2009; Merz and Chapman 2011). Merz and Chapman (2011) assembled an unmanned system that carried a 2.1-kg payload for a 30-min flight. Zarco-Tejada et al. (2009) imaged 0.6 ha of citrus orchards using hyperspectral and infrared cameras. Beside vehicles for positioning instruments, the challenges to advance FBP include a high-throughput analysis of plant samples,

Fig. 11.2 High-clearance prototype FBP tractor in operation over young cotton plants at Maricopa, AZ, for measurement of canopy height, temperature, and spectral reflectance at three bandwidths (White et al. 2012)

management of data flow and analysis, and integrated management of FBP. While all of the six components of an FBP system are not in place, apparently, numerous potential solutions are ready. However, efforts to improve FBP system cannot rely solely on plant scientists but must be integrated across multiple disciplines. A prototype FBP vehicle shown in Fig. 11.2 carried sensors that measured plant height, canopy temperature, and spectral reflectance at three wavelengths (Andrade-Sanchez et al. 2012).

11.3 Root System Architecture

Root systems are fundamental structures for maintaining plant health through mechanical support and water and nutrient acquisition. Root systems have the potential to boost or stabilize yields under abiotic and biotic stress conditions such as in saline, dry, and acid soils, in disease and pest pressure, and in unsustainable fertilizer conditions (Tester and Langridge 2010). Therefore, breeding targets root system traits for crop improvement, especially in challenging environments. Root system architecture (RSA) refers to the complex three-dimensional structure and describes the spatial distribution of age and root types on a single plant (Lynch 1995). RSA differs significantly among species, even among different genotypes of the same species, which allow crop adaptation and exploration in diverse environments (Fitter 2002). Generally speaking, dicots have a relatively simple RSA, while monocots like rice (*Oryza sativa*) or maize (*Zea mays*) have a more complicated RSA (Hochholdinger and Tuberosa 2009). The growth stage is another factor that shapes RSA; the younger plants have less complex root systems, but as plants mature, their root systems become correspondingly more complicated. Modification of root system architecture (RSA) could result in improvements of desirable agronomic traits such as yield, drought tolerance, and resistance to nutrient deficiencies (Beebe et al. 2006; Steele et al. 2007).

Traditional methods of phenotyping RSA were excavation or washed soil cores where one can estimate the total root length, root volume, and average root width of the entire root system (Ostonen et al. 2007). Other common measurements involve measuring the root surface exposed on a soil core or pressed against a transparent surface to estimate root coverage at a certain soil horizon. In both cases, the damage of the topology of the root system and small

numbers of basic measurements can dramatically contribute to the source of bias. In recent years, several newer techniques have been used to nondestructively image root systems to avoid these problems. X-ray computed tomography (Perret et al. 2007) can observe roots grown in soil, but the limitation of X-ray CT system, besides cost, imaging time, and resolution, is the attenuation coefficient, which is typically similar for root and other nonspecific soil organic matters. Perret et al. (2007) developed a protocol for nondestructive visualization and quantification of roots for relatively large soil core using computed tomography (CT) and computer software developed to isolate and analyze the CT matrices. This nondestructive approach revealed details that are not possible to obtain with invasive techniques. Magnetic resonance imaging (MRI) techniques apply the phenomenon of nuclear magnetic resonance (NMR) to image protons of water to convey 3D structural information in a nondestructive manner (Jahnke et al. 2009). Due to the sensitivity of MRI to the soil water content, the resulting image distortion increases the difficulty in analyzing the underground root system compared to using MRI to image the aboveground parts. NMR is also very sensitive to the type of media used for plant growth. Shou and Luo (2009) indicated that, with 88.5 % water content condition, only those portions of the root system greater than 1 mm in diameter can be clearly imaged by MRI. To address this problem, a method complementary to MRI called positron emission tomography (PET) was introduced based on detecting positron-emitting radionuclides such as ^{11}C. PET has the capacity to measure and image the transport and distribution of ^{11}C-labeled photo-assimilates in plants in 3D as well. Jahnke et al. (2009) reported 3D maize root architecture through MRI-PET techniques. A 3D laser scanning technique adopted from Geosciences is described as the best available technique for measuring the surface and shape of roots. Gärtner and Denier (2006) demonstrated a 3D laser scanning device to acquire the structure of the whole tree root system. However, since the laser cannot penetrate opaque growth media, it requires the excavation of the root system in order to expose the target root system. Therefore, using a 3D laser scanner is a destructive technique where the root systems are physically excavated and washed; smaller roots (approx. 1 mm) can be damaged or lost (Gartner et al. 2009) resulting in deterioration of the accuracy of the overall measurements. Fang et al. (2009) applied a transparent 3D root growth system combined with 3D laser scanner to nondestructively study soybean and rice RSA without any contact or perturbation of the root system or the growth medium, but it requires longer imaging times and can be expensive. 3D imaging methods have also been developed for plant root systems grown into transparent gel media. Iyer-Pascuzzi et al. (2010) described a high-throughput and cost-effective platform for phenotyping RSA that combines optical 3D imaging of gel-grown plants with automated image analysis. By measuring large phenotypic variation among 12 rice varieties through the platform, the authors ranked traits for their ability to distinguish genotypes.

Clear tubes installed in the soil at angles ranging from 0 to 90° relative to the soil surface have been used to insert cameras and capture images below the soil surface, known as minirhizotrons. Minirhizotrons have been used to capture root system images over time to determine RSA changes throughout the growing season of various crops, including maize (Aiken 1992; Liedgens 1998; Linsenmeier et al. 2010; Schröder et al. 1996; Upchurch and Ritchie 1983). The difficulty however lies in the image analysis which relies heavily on manual identification of the roots in the image. There have been some recent successes at automating the image processing to characterize the RSA (Zeng et al. 2010). Advances in phenotyping RSA have been achieved, but the ultimate goal is the ability to examine RSA in the field. However, current methods for phenotyping RSA in field-grown plants lack resolution and throughput. The most promising approaches for high-resolution and high-throughput RSA phenotyping as claimed by GrassRoots Biotechnology at Duke University (Zhu et al. 2011) are CT imaging and gel-based imaging platforms. Since CT imaging

has the advantage of being applicable to field-grown plants, gel-based methods are more economical and allow for higher throughput. Concurrent with the advances in 3D imaging and image analysis, software are required to confine important spatial characteristics of RSA.

11.4 Physiological Functional Traits

In crop breeding, the goal is to maintain or increase yield under biotic or abiotic conditions. In a classical model (Passioura 1977), crop yield (Y) is proportional to the total amount of water (W) transpired by the crop and lost through the soil. Water-use efficiency (WUE) or transpiration efficiency (TE) is the ratio of total dry matter produced per unit of seasonal evapotranspiration, and harvest index (HI) is the ratio of yield to aboveground biomass. At the leaf scale, WUE represents a ratio of net CO_2 assimilation and transpiration (Sinclair et al. 1984). Water stress decreases relative water content (RWC) and water potential, stomatal aperture and conductance, and photosynthetic rate (Lawlor and Cornic 2002). Stomatal closure is a major limitation of intercellular CO_2 concentration that reduces photosynthetic rate, with nonstomatal limitation such as reduction in activities of photosynthetic enzymes (Galmes et al. 2011).

A common "surrogate" trait to identify drought tolerance in crops with reproducible indicator of transpiration efficiency in plant physiology is carbon isotope discrimination ($D^{13}C$) measurements (Hall et al. 2010). $D^{13}C$ or stable C isotope ratio ($^{13}C/^{12}C$) in plant represents the photosynthetic efficiency of CO_2 diffusion into intercellular space and the biochemical machinery that actively uptake CO_2 (O'Leary 1988). Plants discriminate against the incorporation of heavier isotope (^{13}C), and this difference is the basis for the sorting of plant photosynthetic efficiency (Farquhar et al. 1989). Isotope ratio mass spectrometry (IRMS) has been the conventional method for measuring isotope ratios, with accuracy that can reach better than $0.1^{o}/oo$ for $^{13}C/^{12}C$ ratio (Vaughn et al. 2004). Samples, such as fully expanded leaves from the main stem, are randomly selected, frozen dried, and ground to powder. Samples are then subjected to a dynamic flash combustion elemental analyzer to convert to CO_2 gas, which is introduced into an isotope ratio mass spectrometer. Stable isotopic fractionations are measured relative to standard (fossil belemnite) and expressed as $D^{13}C$ in parts per thousand ($^{o}/oo$), where $D^{13}C^{o}/oo = \left[\left(^{13}C/^{12}C_{sample}\text{-}^{13}C/^{12}C_{standard}\right)/\left(^{13}C/^{12}C_{standard}\right)\right] * 1,000$ (Farquhar and Richards 1984). The international standard of expressing stable isotope ratio is Pee Dee belemnite (PDB), which has a $^{13}C/^{12}C$ ratio value of 0.0112372 (Craig 1957). In plants, $D^{13}C$ is a measure of transpiration efficiency (TE) since it is directly proportional to atmospheric CO_2 partial pressure (p_i)/sub-stomatal CO_2 partial pressure (p_a), a ratio that is represented by the relationship between photosynthetic assimilation (A) and stomatal conductance (g_s) (Werner et al. 2012), and $D^{13}C$ values are significantly correlated to stomatal conductance and transpiration efficiency in breeding programs (Richards et al. 2010). The advantage of this approach is that samples can be collected at the end of the growing season and $D^{13}C$ values represent the entire plant growth period. The disadvantage is the relative high cost per sample (between US $10 and $30), and $D^{13}C$ values have to be normalized to "best treatment" controls to determine the best plant selection.

Chlorophyll fluorescence measurement methods provide powerful tools for the nondestructive evaluation of photochemical and nonphotochemical function in chloroplast systems in plants and have been utilized in crop production as a measurement of plant photosynthetic capacity in optimal development condition and under stress. Chlorophyll content or density in leaves represents the efficiency of the conversion of light energy to stored biochemical energy that can be utilized for growth and acclimation to adverse environment (Carter and Knapp 2001) and provides an indirect estimate of nutrient status by the incorporation of nitrogen (N) (Filella et al. 1995). The ability of plants to overcome drought stress is a correlation of chlorophyll

system to maintain functionality under drought stress.

Pulse amplitude modulation (PAM) fluorimetry along with the saturation pulse is the method that has been used in photosynthesis-related stress physiology studies (Schreiber 2004). Chlorophyll fluorescence results from light energy that is not absorbed by the photosynthetic system, and the dissipation of excess energy results in increased fluorescence, such as in the case of drought. PAM fluorimeters measure relative quantum yield of chlorophyll fluorescence by applying a light energy with constant pulse amplitude. Fluorescence emission competes with a number of phytochemical pathways, and the relative fluorescence yield ratios of F_v/F_m between controls and treatments indicate stress or altered function of the photosynthetic system (Adams and Demmigs-Adams 2004). The saturation pulse method allows the evaluation of photochemical and nonphotochemical quenching parameters as well as estimates of quantum yield of energy conversion in photosystem II. A short burst of saturating radiation during induction phase results in a complete saturation of the photochemical pathways and an over-reduction of photosystem II components (Rohacek and Bartak 1999). PAM fluorimetry has been utilized as a noninvasive approach to evaluate different enhanced performance traits in *Phaseolus* (Rascher et al. 2011).

Hyperspectral imaging spectroscopy (discussed in detail in Chaps. 4 and 6) can be a very valuable tool in plant stress phenotyping due to the availability of many affordable camera systems (Romer et al. 2012). Hyperspectral imaging deals with measurements of radiative property of plant leaves and canopies which can be used to determine plant physiological or physical traits. Leaf spectral reflectance is characterized by low reflectance in the visible range (400–700 nm) due to strong absorbance properties of photosynthetic pigments, high reflectance in the infrared range (700–1,100 nm) due to the scattering of light by leaf mesophyll, and high reflectance in shortwave infrared range (1,100–2,500 nm) by reflectance properties of lignin content, cellulose, protein, and water (Rascher et al. 2010). Remote sensing utilizing hyperspectral imaging provides timely assessment of crop growth conditions during the growing season. A number of vegetation indices have been developed from imaging data to detect plant physiology status: (1) enhanced vegetation index (EVI) with blue reflectance and sensitive to canopy structural variations (Huete et al. 2006), similar to normalized difference vegetation index (NDVI) which is sensitive to chlorophyll color range (Myneni et al. 1995), (2) red edge inflection point (REIP) measuring the slope of red absorption (Penuelas and Filella 1998), (3) photochemical reflectance index (PRI) which detects the epoxidation state of pigments in the xanthophyll cycle in the range of 531 and 570 nm (Penuelas et al. 1995), (4) normalized difference nitrogen index (NDNI) with nitrogen absorption at 1,510 nm (Serrano et al. 2002), (5) normalized difference lignin index (NDLI) with lignin absorption at 1,754 nm (Serrano et al. 2002), (6) cellulose absorption index (CAI) with cellulose absorption between 2,000 and 2,200 nm (Nagler et al. 2003), (7) plant senescence reflectance index (PSRI) which correlates senescence and fruit ripening (Merzlyak et al. 1999), (8) carotenoid reflectance index (CRI1 and CRI2) in the yellow spectrum region with CRI1 correlating with carotenoid and CRI2 with chlorophyll ratio (Gitelson et al. 2002), (9) anthocyanin reflectance index (ARI1 and ARI2) in the yellow and red spectrum regions with ARI1 correlating with anthocyanin and ARI2 with chlorophyll ratio (Gitelson et al. 2001), and (10) normalized difference water index (NDWI) which correlates with canopy water content (Gao 1996). Since hyperspectral reflectance spectroscopy relies on solar radiation from sunlight as a source of measurement, hyperspectral data can vary significantly when comparing different plots and genotypes depending on the consistency of sunlight during measurement, time of measurement, or angle of solar energy (Jones and Vaughan 2010).

11.5 Phenotyping of Chemical Traits Related to Quality and Nutritional Value

The quality and nutritional value of grains obtained from crop plants are related to chemical traits that are functionally involved in the growth and development of crop plants. Although the phenotyping of the quality and nutritional traits has been discussed in Chap. 15, here these traits have been briefly discussed as functional traits in general. Depending on the analysis technique employed, chemical trait analysis can be classified into destructive and nondestructive. For example, peanut seed oil content can be measured by ether extraction (destructive) or nuclear magnetic resonance (nondestructive). If the method of analysis is nondestructive, the sample extraction step can be omitted. Nondestructive methods not only save plant samples (such as seeds) but also reduce the analysis cost by eliminating the extraction step.

For the destructive method, the extraction efficiency varies. Castor seed oil was extracted by a hydraulic plant press (Fig. 11.3). Crude oil was extracted by the press, but the extraction process was very slow. On an average, four people for 2 weeks can only process 50 samples (two replicates for each sample). The extraction results were also affected by room temperature in the lab and the pressure provided by the man power. The ether extraction using ANKOM XT15 Extractor (Fig. 11.4) is another destructive method. Peanut seeds (~5 g) were baked at 130 °C for 6 h and then put in a sealed jar before extraction. Seeds were ground in a coffee bean grinder into a fine powder. The ground powder (1.5 g, MB = mass of seeds before extraction recorded) was transferred into an XT4 filter bag (ANKOM Technology). The filter bag was sealed with a heat sealer and was then inserted into a metal coil. The ground powder was extracted with ether at 90 °C for 30 min in the ANKOM Extractor. The ether-extracted oil was collected in the bottom of the extraction cylinder with defatted seed (MA) remaining in the bag. The oil content can be calculated using the formula of % oil = (MB−MA)/MB. Compared with the hydraulic plant press method, the efficiency of ether extraction method was doubled. The nondestructive methods (such as

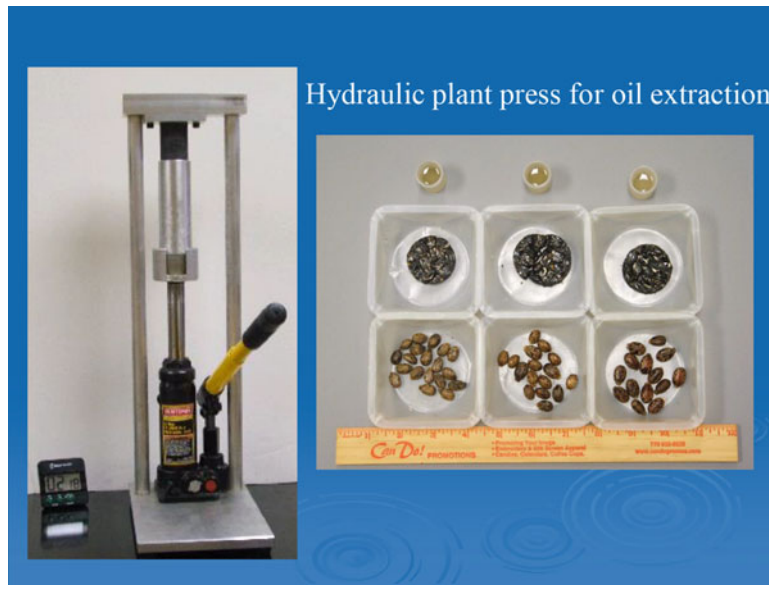

Fig. 11.3 Castor oil extraction by hydraulic plant press

Fig. 11.4 Peanut oil extraction by ether solvent using ANKOM XT15 Fat Extractor

NMR and NIR) for oil measurement will be desirable and have been described in the following section.

For other compound extractions (such as resveratrol), some additional cleanup steps may be required prior to analysis. Resveratrol exists or accumulates in a low concentration in plants but plays important roles for plant protection and contributes to human health. For the extraction of resveratrol, the method used was destructive with a long process and also needed specific attention. Under bright light, *trans*-resveratrol can easily convert to *cis*-resveratrol; therefore, all the procedures for sample preparation were performed under a yellow light (Sanders et al. 2000). Approximately, eight grams of air-dried seeds were ground into a fine powder in a coffee grinder. Ground seed tissue (3 g) was transferred into 50-ml Falcon tubes and homogenized with 9 ml of 80 % ethanol using a PowerGen 125 homogenizer (Fisher Scientific). The homogenized samples were centrifuged (Eppendorf, 5415D) at 12,000 rpm for 3 min. Two milliliters of supernatant was taken and cleaned by solid-phase extraction using a Poly-Prep chromatography column (0.8 × 4 cm, Bio-Rad) packed with ~1 ml mixture (1:1 w/w) of Al2O3 (EM Science, Gibbstown, NJ) and silica gel 60 RP-18 (EMD Chemicals Inc., Gibbstown, NJ). The packed column was conditioned with 80 % ethanol. The supernatant was applied to the equilibrated column, and the effluent was collected into a 4-ml vial. The column was washed with an additional 2 ml of 80 % ethanol, and the effluent was collected into the same vial. The collected solvent was evaporated at 50 °C to dryness with a nitrogen gas stream. The extracted compounds were dissolved in 1 ml of 20 % acetonitrile and filtered (at 0.45 μm filter) prior to injection for HPLC analysis. Resveratrol extraction process was really labor intensive. One person may only extract about 30 samples a day.

For any chemical analysis, the employed methods have to be selected. Selectivity of a method refers to the extent to which it can determine particular analyses in a complex mixture without interference from the other components

in the mixture. For method selection, we have to consider and balance the following factors: sensitivity, accuracy, precision, and repeatability as well the cost and throughput. Sensitivity is the difference in analysis concentration corresponding to the smallest difference in the response of the method that can be detected. Detection of compounds with a low concentration in the sample may require a highly sensitive HPLC method (such as detection of average 0.5 μg/g *trans*-resveratrol in peanut seeds). If the method employed is beyond the limit of detection, the amount of *trans*-resveratrol may not be detected for distinguishing different peanut samples. Accuracy is a measure of the closeness of the experiment value to the true amount of compound in the sample. The method employed should get the experiment value as close as possible to the true value. Precision is a measurement of closeness of individual experiment values to each other. Variation in individual experiment values should be expected and permitted. Repeatability is a type of precision relating to measurements made under repeatable conditions (i.e., using the same method and material conducted by the same operator within the same laboratory). Precision does not necessarily mean accuracy. For a good method, the experiment value should be repeatable within and among the laboratories.

Using known amounts of compounds to establish the standard curve is the first step for compound detection and quantification. A good linearity from five different standards is usually sufficient for producing a calibration curve. If the linearity is poor, more standards may be required. The time-domain pulsed NMR method is an AOCS (American Oil Chemists' Society) recommended standard method for rapid and simultaneous determination of oil and moisture content in oil seeds. Peanut oil content was quantified by NMR analysis. For the measurement of peanut oil content in seeds, nine standards were prepared by weight. For each standard, shredded paper was added to the sample tube to serve a matrix, along with a carefully measured mass of oil. The peanut seeds in the tube were transferred into an NMR machine with a magnetic field. The NMR was maintained at 40 °C and operated at a resonance frequency of 9.95 Mhz. In a magnetic field, certain atomic nuclei resonate at specific radio frequencies. This resonance can be converted into a signal and measured to determine the amount and nature of the particular nuclei in a sample. Solids and liquids containing hydrogen nuclei can easily be distinguished in this manner. Oils and water can be differentiated by employing specifically timed radio pulse, provided the moisture content is relatively low (<15 % of the total mass). For each signal acquisition, spin-echo parameters consisted of a 90° pulse of 10.44 μs and reading at 50 ms followed by a 180° pulse of 21.38 μs and reading at 7 ms. A 2-s recycle delay between scans was used, and a total of 16 scans were collected for each sample. Detected signal can be calibrated against known standards and calculated into oil content. After oil content measurement, the peanut seeds can still be saved or used for other chemical analyses or field planting. Compared with hydraulic plant press and ANKOM Fat Extractor methods, the NMR analysis for oil content measurement is 32 times and 16 times efficient, respectively. One person can measure 200 samples for oil content within a week without any cost. Near-infrared (NIR) technology can also be used for measuring oil and moisture contents, but the method has not yet been recommended by AOCS(American Oil Chemists' Society) for oil seeds (Baianu et al. 2012).

For HPLC analysis of some compounds, certain detector, column, and mobile phase may be required. For resveratrol detection, separation of metabolites was performed on RP-HPLC system (Agilent 1,100 series) using a C18 column (4.6 × 150 mm, 5 μm, Agilent Technologies) at 40 °C with a binary pump and autosampler. The mobile phase consisted of A, filtered sterile water containing 0.1 % formic acid at pH 2.5, and B, HPLC grade acetonitrile. The flow rate was 1.5 ml/min with the following gradient: 10 % B for 2 min, 10–30 % B for 8 min, 30 % B for 1 min, followed by a column wash at 95 % B for 6 min, and 10 % B for 9 min before the next injection. The volume for sample injection was

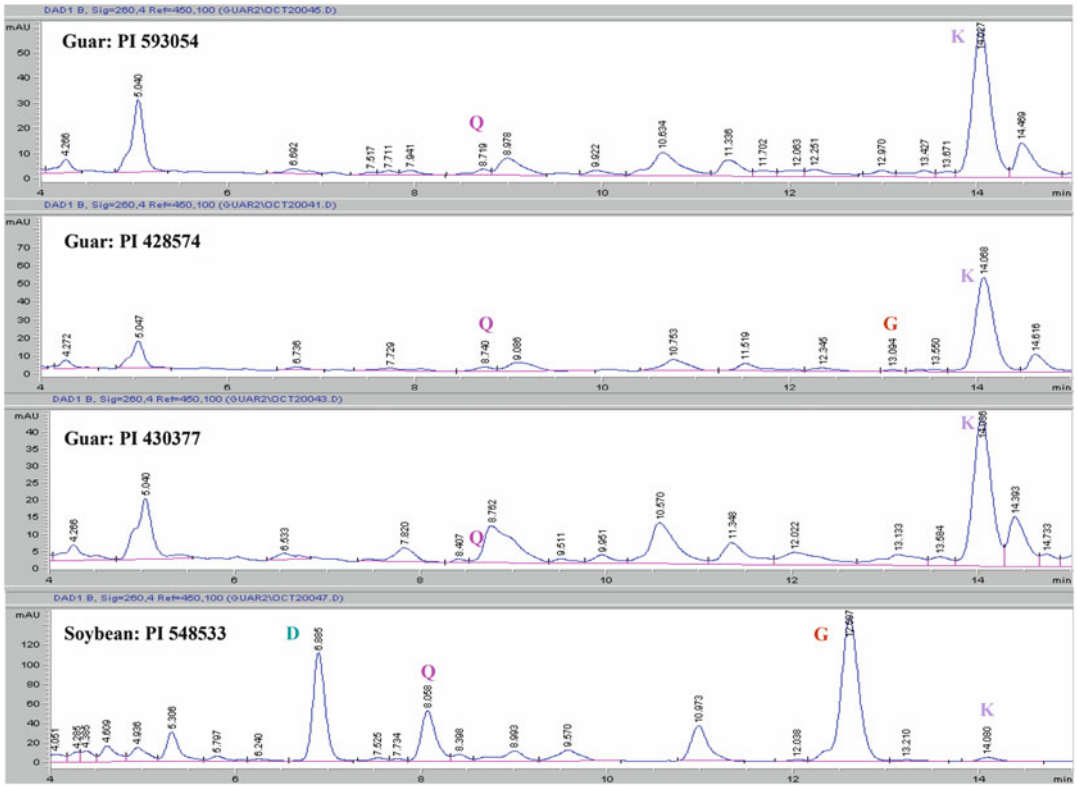

Fig. 11.5 A chromatogram of phytochemicals separated at 260 nm on the HPLC system. *X* axis represents for retention time (min), *y* axis for million absorbent unit (mAU), *D* for daidzein, *G* for genistein, *Q* for quercetin, and *K* for kaempferol

30 μl, and the analytes were monitored with a diode array detector (DAD) at 310 nm absorbance. *trans*-Resveratrol in the extract of each accession was quantified at 310 nm by referencing the peak area of an external authentic standard of resveratrol.

If several compounds can be detected from the same extraction, the efficiency of extraction, detection, and quantification will be greatly increased. For example, daidzein (D), genistein (G), quercetin (Q), and kaempferol (K) can be detected from the same extraction in the guar seeds (Fig. 11.5, Wang and Morris 2007). The big challenge for HPLC analysis is that some peaks are clearly identified but we may not know for what compounds the peaks represent. More research work (such as HPLC-mass spectrometry) is required to finally identify the compounds.

11.6 Postharvest Characteristics

Objective measurements of in-season plant responses and the resulting postharvest characteristics are essential in identifying the genetic control of plant response to stress. Postharvest characteristics such as size and maturity distribution, nutritional and chemical composition, and flavor characteristics are strongly influenced by a combination of genetic potential and environmental stresses. Spectral characteristics of seeds after harvest have been successfully used to identify and separate genetically different seeds. Armstrong et al. (2006) utilized near-infrared reflectance (NIR) to measure wheat and flour characteristics. Dowell et al. (2009) utilized NIR to sort waxy wheat from non-waxy wheat and insect-infested grain

from un-infested grain. The desirable trait of high ratio of oleic to linoleic fatty acid is the result of a double recessive gene. Determining the ratio of oleic to linoleic fatty acids in peanut seeds has generally been destructive (Chamberlin et al. 2011). Dean et al. (2013) showed that the development of oleic fatty acid in peanuts may be related to kernel maturity as well as genetics. Nondestructive screening of individual seeds has been done with limited success (Sundaram et al. 2011).

In an indeterminate crop such as peanut, a single plant will have seeds that are at various stages of physiological maturity due to continuous flowering and fruit addition in response to environmental conditions. In response to spatially variable conditions such as soil moisture and fertility and rainfall, there may be a considerable spatial variation in peanut maturity as well, resulting in a stochastic distribution of peanut maturity at harvest (Fig. 11.6). Williams and Drexler (1981) observed that the middle layer or the mesocarp of the peanut hull varied in color from white to yellow to orange to brown and black as the peanuts matured. As a result, they developed the method known as the hull scrape, whereby the outer layer or exocarp of the peanut hull was removed exposing the mesocarp of the freshly dug peanut pods. After removal of the exocarp, the peanuts are then sorted according to color, and a prediction of days to harvest is made. Various attempts have been made to remove the human subjectivity in classifying the peanut maturity profile using colorimeters (Grimm et al. 1998) and computer-assisted image analysis (Boldor et al. 2002; Colvin et al. 2013). Other techniques such as nuclear magnetic resonance (Tollner et al. 1998), seed-hull weight ratio (Pattee et al. 1977), and measurement of arginine (Johnson et al. 1976; Hammons et al. 1978) have been used to determine the maturity of peanuts prior to harvest. All of these methods require destructive sampling. Rowland et al. (2008) indicated that changes in the NDVI of a peanut canopy may be useful in predicting peanut maturity prior to digging.

Fig. 11.6 Typical maturity distribution of peanuts based on the color distribution of the mesocarp

The maturity distribution is largely controlled by environmental conditions such as the availability of water, air temperature and humidity, and soil temperature (Padmalatha et al. 2006); however, the relative maturity among peanut types (runner, Spanish, Valencia, and Virginia) and cultivars within a market type is controlled by genetics. For instance, Spanish-type peanuts, generally, are more determinate in their flowering and fruiting patterns.

Measuring seed size distribution and hull to kernel ratio in peanut typically requires that a sample of peanuts be shelled and the kernels physically sized over a series of screens (Davidson et al. 1982). However, a system using nondestructive imaging using X-ray has been developed and tested to accurately measure the size distribution of peanut pods and the kernels within the pods and the kernel to hull ratio (USDA, ARS, National Peanut Research Laboratory, unpublished data 2008–2013). Similarly, peanut kernels must be shelled to accurately measure their kernel moisture content. Kandala and Nelson (2005) and Trabelsi and Nelson (2006) have developed instruments to measure the kernel moisture content while still in the hull by measuring the dielectric constant at radio and microwave frequencies, respectively. Schmilovitch et al. (1996) used similar techniques to measure the moisture content of in-shell pecans.

Researchers have developed methods to estimate the heritability of peanut flavor attributes and then use those methods to identify parental lines to enhance the desired attributes of roasted peanut, sweet and aromatic, while diminishing undesirable attributes such as bitter and astringent (Isleib et al. 2003; Isleib and Pattee 2007; Pattee et al. 2001). However, destructive sampling and highly trained taste panels have been employed to perform these time-consuming flavor analyses.

11.7 Other Functional Characteristics

In-season measurements may consist of physiological measurements such as gas exchange of leaves or sap flow (Rowland et al. 2005); physical measurements of plant structure such as root density profiles, internode length, and node number; or spatial canopy measurements such as leaf temperature and multi- and hyperspectral images. These in-season measurements are often destructive in nature and very labor intensive. Therefore, various imaging techniques have been developed and tested to measure in-season plant characteristics. Payero et al. (2004) found that several spectral indices, including but not limited to the normalized difference vegetation index (NDVI), the infrared percentage vegetation index (IPVI), and the transformed vegetation index (TVI), were sensitive to changes in the height of alfalfa, but only 4 of 11 indices were sensitive to changes in plant height of grasses. Jones et al. (2007) used a combination of multispectral imaging and ultrasonic detection of plant height to estimate chlorophyll content of *Spinacia oleracea* (spinach) plants. Kulkarni et al. (2008) utilized the green normalized difference vegetation index (GNDVI) and spatial regression to determine the response of soybean to infestations of soybean cyst nematode (*Heterodera glycines*). Root growth distribution is difficult to measure using any method; however, capturing images through clear tubes inserted into the soil in the crop row has been used successfully to assess in-season root growth (Taylor et al. 1970; Vos and Groenwol 1987).

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Role of Fluorescence Approaches to Understand Functional Traits of Photosynthesis

12

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Abstract

Chlorophyll *a* fluorescence is a fast, non-invasive, non-destructive tool. Plant responses of the photosynthetic apparatus of leaves and plants can be measured in real time using digital imaging, and this gives the opportunity through analysis of the data to understand more about the growth and developmental processes of plants as they adapt and respond to the changes in the environment. Chlorophyll *a* fluorescence fulfils the criteria to be used in high-throughput (HTP) screening, as long as fundamental rules are being taken care of like good plant preparation and proper use of measuring protocols. Four parameters derived from fluorescence measurements that can be used for HTP screening of plants are being discussed because these parameters can be used as functional traits of photosynthesis. Selecting plants on these properties will offer possibilities in improving the crop yield at field conditions and in greenhouses.

12.1 Introduction

Phenotyping of plants is not an easy task since plant growth and development are dynamic processes which are continuously changed by environmental conditions. Plant physiology is offering many physiological parameters to be applied in the study of abiotic and biotic stress effects on plants in different science fields like horticulture, agriculture and agronomy. Today we have the availability of high-tech instrumentation which can measure many parameters which correlate with plant growth, development and crop yield under biotic and abiotic stresses. These are applied as indicators in plant breeding programmes. These parameters are mostly derived from morphological measurements using digital cameras in the colour and NIR range. Recently, one physiological process has become a lot of attention from plant breeders: photosynthesis (Schurr et al. 2006). Plant breeders are especially interested in this process since the photosynthetic status of plants can be imaged using chlorophyll *a* fluorescence phenotyping technology (Woo et al. 2008; Jansen et al. 2009). By measuring the process of photosynthesis, physiological parameters of the physiological status can be derived from which information can be extracted on the ability

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of plants to deal with changes in the environment (Baker and Rosenqvist 2004). Plants are unique in that they are capable of converting light into energy used in the process of photosynthesis for synthesising starch and sugars. For this reason, this unique property is important to characterise and finally to understand the photosynthetic process better. Increasing the photosynthetic efficiency and the photosynthetic rate will have great impact on the productivity of crops. Light energy that is absorbed by chlorophyll in a photosynthetic process can be distributed into three major different pathways: (a) it can be used in the photosynthesis process, (b) it can be dissipated as heat or (c) it can be emitted as fluorescence (Misra et al. 2012). These three processes are in competition with each other. Since the sum of the energies of the three pathways is constant, this is a fundamental law from physics and called "law of conservation of energy", any increase in the efficiency of one pathway will result in a decrease in the yield of the other two. Determining the intensity of chlorophyll fluorescence by applying specially developed measuring protocols will give information about changes in the efficiency of the photosynthetic process and heat dissipation (Baker 2008; Schreiber et al. 2012). To understand better how these measuring protocols work, one should consider the following. The phenomenon of chlorophyll *a* fluorescence from plants is an intrinsic property of the molecule chlorophyll and not a property of the photosynthetic apparatus. This means that even "dead" plant material will show fluorescence. All processes that lower the chlorophyll fluorescence intensity are defined by the term "quenching". The so-called "dead" plant material will show relatively high fluorescence intensity since no photosynthesis is occurring. Lowering of fluorescence intensity is due to the "quenching": energy is transferred to the photosynthetic process instead of being directed to the thermal and fluorescence pathways (Krause and Weiss 1991). If more energy is directed into the photochemistry pathway, the lower the fluorescence intensity will be. The absorption of photons by antenna molecules is a very fast process and occurs within femtoseconds leading to excited chlorophyll molecules and excited electrons (Tth 2006). These excited electrons are passed on to the reaction centres of photosystem II and used for energy production through photochemistry. As mentioned before, a part of the energy of the excited electron will be used for photochemistry; a part will be released as heat and as fluorescence. If a reaction centre is already occupied by an electron, a new excited electron that arrives at the reaction centres cannot be transferred into the photochemistry pathway. The electron falls back into its initial ground state and the energy gained through the absorption of light is emitted as heat and fluorescence. Heat is being generated via release of energy in small steps through the vibrational states of the molecule and fluorescence in a larger step when it falls back from a higher electronic state to the ground state. If more and more reaction centres are occupied by electrons, more excited electrons will fall back to the ground state. This will produce an increase in the fluorescence intensity. On the other hand, if excited electrons can be quickly channelled into the photochemistry process, the fluorescence intensity will be low. However, chlorophyll *a* fluorescence is a method that indirectly indicates what the status of the photosynthesis process is. Chlorophyll *a* fluorescence correlates with CO_2 photoassimilation and O_2 evolution (Walker et al. 1983; Edwards and Baker 1993; Genty et al. 1989). Not all the energy from the absorbed photon is used for photochemistry. In the best case for a healthy plant, around 83 % of the solar energy is absorbed into photosynthesis about 15 % is released as heat and 2 % as fluorescence. It is important to use the right measuring protocols and the right pretreatments of the plant material. If this is fulfilled, chlorophyll *a* fluorescence is a useful tool to study the effects of environmental stresses on plants since photosynthesis is often lowered in plants experiencing adverse conditions, such as drought, temperature, nutrient deficiency, polluting agents and infections by pathogens for different plant species and within a plant species for different genotypes (Harbinson et al. 1989; Ögren 1990; Groom and Baker 1992; Shavnin et al. 1999; Chaerle and van der Straeten 2001;

Sinsawat et al. 2004). Therefore, measurement and analysis of chlorophyll *a* fluorescence parameters is considered as an important methodology for evaluating the health or status of the photosynthetic process within a leaf or the whole plant (Krause and Weiss 1991; Clark et al. 2000). The common method for measuring fluorescence signals from plants is a spot measurement which measures a small part of the leaf. Other parts of the leaf have to be measured in a sequential order. Furthermore, these measurements are carried out by hand and this is laborious, since the sensor has to be positioned manually onto the plant for each position. Chlorophyll fluorescence imaging can measure a whole plant simultaneously in one single measuring protocol, remotely and without manually touching the plant. The total measurement requires only a few seconds per plant.

In this chapter the focus will be on the application of chlorophyll fluorescence for high-throughput screening systems. This yields a constraint on the time that can be used for the fluorescent measurement. The time of measurement should be as short as possible in order to be able to measure large amounts of plants, in the order of 1,000 per day, and also to minimise the influence of the measurement on the photosynthesis and to minimise the time needed for the transportation of the plants out of the growing area to the station where the plants are being measured. This will maximise the time that the plants are at their growing conditions. For this reason a fluorescent measurement should not last more time than several seconds per plant.

12.2 Phenotyping Plants Using Chlorophyll *a* Fluorescence

An important trait of photosynthesis is the rate of photosynthesis. This parameter is directly correlated with the increase of biomass of plants. Plant production could be improved by the selection of varieties and cultivars that perform highest on the photosynthetic rate at optimal and also at adverse climate conditions. Under field conditions, selection of these plants has to be made on high and low irradiance, large temperature regime, tolerance to salinity and drought. These tolerances to environmental conditions are of high importance to be able to maintain and increase crop productivity. Under climatised conditions like in greenhouses, selection on plants can be made under optimised day length, light intensity, temperature and CO_2 concentration. Therefore, measuring the rate of photosynthesis has become an important field of study and a real challenge to be measured in high-throughput screening. The photosynthetic rate can be measured as the exchange of CO_2. This is directly related to the chemical reaction equation of photosynthesis: $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$ (Rabinowitch and Govindjee 1969). Under the absorption of light, the photosynthetic process is used solar energy to convert carbon dioxide and water into carbohydrates and oxygen. However, this uptake of CO_2 is quantified by measuring the uptake of CO_2 as a decrease of this gas in a small chamber that contains a part of a leaf or in some cases the whole plant (Ehleringer and Björkman 1977). This measurement is time consuming, laborious, cannot be measured at a high scanning rate per plant and not remotely. For this reason, an alternative technology has to be used to fulfil the constraint of high-throughput (HTP) screening of plants. A parameter that correlates with the maximum efficiency of photosynthesis of PSII is F_v/F_m (Butler 1978). This parameter is considered as an important trait of photosynthesis, because it gives an estimate of the highest achievable efficiency of PSII photochemistry. It yields the maximum value for the percentage of absorbed light that is used for PSII photochemistry. Any biotic or abiotic influence or stress that has direct or even indirect interaction with the photosynthetic process can lower this efficiency. A second important parameter is the parameter that correlates with the photosynthetic rate: the effective or ongoing efficiency of photosynthesis, F_q'/F_m' (Genty et al. 1989). This parameter is used as an important trait of photosynthesis, because it gives an estimate of the photosynthetic rate of plants at the given irradiance and environmental conditions. This yields quantitative

Table 12.1 Overview of the major fluorescence parameters and equations that are usable in HTP screening using digital imaging

Parameter	Definition	Physiological relevance	Measured/calculated from equation
F_o	Minimum fluorescence of dark-adapted plants	All PSII reaction centres are open	Measured
F_m	Maximum fluorescence of dark-adapted plants after a saturating light pulse	All PSII reaction centres are closed due to the saturation pulse	Measured
F_v	Variable fluorescence of dark-adapted plants	Shows the maximum performance of PSII on photosynthesis	Calculated as F_m–F_o
F_v/F_m	Variable fluorescence normalised by the maximum fluorescence of dark-adapted plants	Correlates with the maximum efficiency at which light absorbed by PSII is used for photosynthesis	Calculated as $(F_m–F_o)/F_m$
F_t'	Fluorescence at time point t of light-adapted plants	Part of the PSII reaction centres are open, rest is closed	Measured
F_m'	Maximum fluorescence of light-adapted plants after a saturating light pulse	All PSII reaction centres are closed due to the saturation pulse	Measured
F_q'	Variable fluorescence of light-adapted plants	Shows the effective performance of PSII on photosynthesis at given photosynthetically active photon flux density, PPFD	Calculated as $F_m'–F_t'$
F_q'/F_m'	Variable fluorescence normalised by the maximum fluorescence of light-adapted plants	Correlates with the efficiency at which the absorbed light is being used for photosynthesis at the given PPFD	Calculated as $(F_m'–F_t')/F_m'$
NPQ	Non-photochemical quenching	Monitors the heat loss from PSII	Calculated as $(F_m–F_m')/F_m'$
rel-ETR	Relative electron transport rate	Measure for the electron flow that correlates with the photosynthetic rate	Calculated as $((F_m'–F_t')/F_m')$* PPFD ×*α_L*PSII/PSI

information on the performance of plants at the given conditions. The third trait of photosynthesis that will be discussed in this chapter is the non-photochemical quenching, NPQ (Bilger and Björkman 1990). This parameter correlates with the energy that is not used in the photosynthesis but is converted into heat or thermal energy. A higher value indicates that less energy is going into the photochemistry and more into the thermal pathway. The final parameter is the relative electron transport rate, rel-ETR, and is a better estimate of the photosynthetic rate, because it takes into account the absorbed light by the plant. By multiplying the effective photosynthetic efficiency by the irradiance that is used in photosynthesis or photosynthetic photon flux density (PPFD), a measure for rel-ETR can be established (Walker et al. 1983; Genty et al. 1989; Edwards and Baker 1993; Fryer et al. 1998). The name refers to the electron transport rate which is directly linked to the exchange of CO_2 and therefore the photosynthetic rate. In the next three paragraphs, an introduction is given on the induction curve of fluorescence, followed by several fluorescence signals that can be measured in a short time interval from which these above-mentioned parameters are calculated for high-throughput phenotyping for plants in the dark, on so-called dark-adapted plants, and plants that are subjected to light, light-adapted plants (Table 12.1).

12.3 Fluorescence Induction: The Basics of the Measurement

It was already suspected for a long time that the chlorophyll fluorescence intensity during the induction period is a measure for the ongoing photosynthesis of plants (Kautsky and Hirsch 1931; McAlister and Myers 1940). Kautsky and Hirsch (1931) were the first who realised this correlation. Their one-page article describes that when chlorophyll is being illuminated by a constant light, fluorescence of changing intensity is being emitted. The intensity of the fluorescence as observed by their eyes first rises rapidly from weak intensity to a maximum value and then decreases slowly to a steady state level of low intensity. They concluded that the slow decrease in fluorescence from the maximum value to low fluorescence correlated with the observations done by Warburg (1920) on the induction time needed to establish a constant O_2 production (after about 4 min): after a first slow increase in the rate in O_2 evolution, the rate in O_2 evolution became constant. This correlation could be useful to study the photosynthetic process by measuring the fluorescence intensity, instead of the evolution of O_2 or the uptake of CO_2. Using better equipment in the 1960s until 1980s, the induction curve was used extensively to study the process of photosynthesis (see for reviews: Govindjee 1995; Strasser et al. 2000). It was soon recognised that the fast fluorescence induction rise in the first second could be used to measure the maximum efficiency of photosynthesis (Fig. 12.1). This method uses a high intensity of constant irradiance to induce photosynthetic activity and to saturate the photosynthesis. At the same time, this saturating light is used to measure the resulting fluorescence. The resulting fluorescence is measured using a photodiode or digital CCD camera. In the first 50 µs F_o, fluorescence at the origin is being measured and depending on the used speed of the electronics, the induction curve is being measured at different time points, ending in the maximum intensity of the fluorescence, F_m. This method is called direct fluorescence (Strasser et al. 2000). In the 1980s a new measuring protocol was developed by Schreiber et al. (1986): pulse-amplitude modulated fluorometer, PAM (Rohacek and Bartak 1999). This new measuring

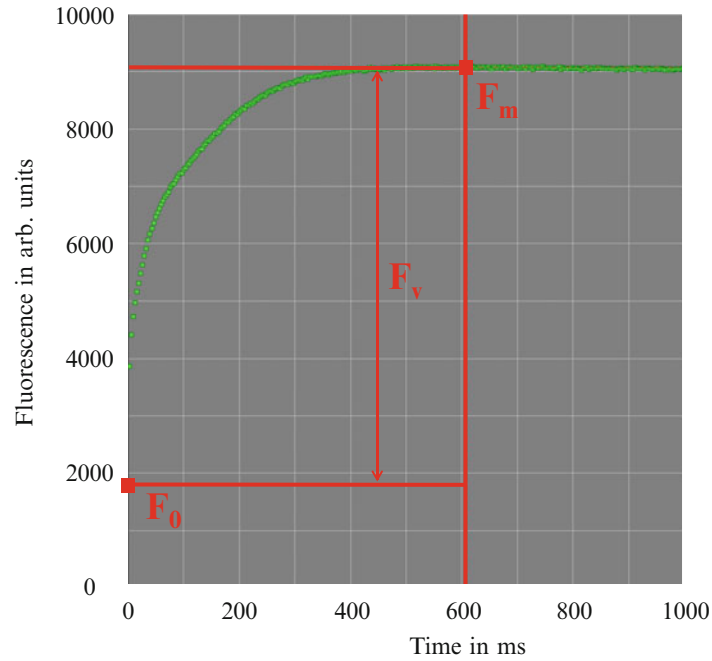

Fig. 12.1 Example of an induction curve of a dark-adapted plant using the direct fluorescence method measured at a measuring light intensity of 1,500 µmol m^{-2} s^{-1} and at a sampling rate of 380 samples/s

device works on the principle of a modulated weak measuring beam that is low enough in intensity not to close the reaction centres of dark-adapted plants. A modulated excitation light results in a modulated fluorescence signal. This fluorescence has the same frequency as the modulated excitation light, and this information is used to discriminate the measuring modulated fluorescence signal from the background signal. On dark-adapted plants F_o is first being measured. Then a saturating pulse of constant intensity is applied to induce photosynthetic activity in order to close the reaction centres. In the same way as for the direct fluorescence method, the induction curve can be measured, but what it is used most frequently for is the value of F_m. In the presence of light, so-called actinic light, these measurements can also be performed yielding the photosynthetic parameters in the light. More methodologies were developed; all with the purpose of measuring the photosynthetic parameters. These protocols can roughly be classified into either direct fluorescence or modulated fluorescence. The main advantages of the modulated light systems are the real-time subtraction of the background light and flexibility in using different light sources for the measuring and saturating light (Schreiber et al. 1986, 2012). The main advantages of the direct fluorescence method are the better signal to noise and its ease of use (Loriaux et al. 2013). Evaluating the different methods to measure the photosynthetic parameters, comparable results were obtained (Koblizek et al. 1999; Suggett et al. 2003; Röttgers 2007). However, induction curves yield sometimes more information than static F_v/F_m measurements. Anaerobic treated leaves of *Arabidopsis thaliana* showed increasing differences in the fluorescent transients at increasing anaerobic conditions, while the F_v/F_m value remained constant (Nellaepalli et al. 2012).

12.4 Dark-Adapted Plants: Description of the Fluorescence Parameters

A plant coming from the light has reaction centres that are open for capturing new incoming photons and reaction centres that are closed, because they just have captured photons. As the plant makes a transition from light to dark, no new incoming photons are available anymore and for the already captured photons, the photosynthetic process needs time to downregulate to the state when all the reaction centres are open. This usually takes about 30 min. Shorter adaptation times can yield lower F_v/F_m values (Schansker et al. 2005). However, if a plant has been subjected to stress, this can take a much longer time, up to 24 h (Rascher et al. 2000; Maxwell and Johnson 2000; Qiu et al. 2003). It is advisable to test a range of dark-adaptation periods to check that the F_v/F_m value cannot be increased by using longer dark-adaptation times. Sometimes it is advised to measure the plants after a dark period during the night. This is called predawn dark adaptation. The dark-adapted state of a plant is the state at which all reaction centres are open (Baker and Rosenqvist 2004). Once the plant is dark adapted, two fluorescence signals can be measured: F_o and F_m.

F_o: the minimum fluorescence or fluorescence at the origin is measured at measuring conditions that do not close the reaction centres (Schreiber et al. 1986). The direct relation of this parameter F_o with the photosynthesis process is that when the photosynthesis is operating at maximum efficiency, the fluorescence intensity is minimised (Duysens and Sweers 1963). When the photosynthesis is not operating at full efficiency, F_o can have a fluorescence intensity between the minimum, F_o without stress, and maximum, F_m, possible values. Unfortunately, F_o has no functional trait for photosynthesis when using fluorescence digital CCD-camera systems. The fluorescence intensity as measured with a digital CCD camera depends among others on (1) the amount of chlorophyll on a particular spot, more chlorophyll will yield a higher value for F_o, (2) the intensity of the measuring beam, which normally decreases in intensity with the square of the distance, (3) the distance between the particular spot and the lens of the camera that decreases the fluorescence intensity with the square of the distance and (4) the angle of the particular spot on the leaf with respect to the measuring beam. A larger leaf angle will decrease the effective light

intensity on the leaf. This implicates that it is not clear if differences in F_o are being measured and are the results from the status of the photosynthesis, from instrumental layout or plant morphology. For this reason, the measurements can only be compared by taking ratios of the fluorescence intensities. They have to be calibrated or normalised to each other. This is accomplished by measuring the F_m value. This F_m value is also needed to calculate the maximum efficiency of photosynthesis. Therefore, measurement of F_o and F_m for all the different spots or pixels of a digital CCD camera will yield different intensities for F_o and F_m for the above-mentioned reasons of instrumental layout and plant morphology, but in the calculation of the maximum efficiency of photosynthesis, fluorescence signals are being normalised on F_m values for each pixel with absolute values ranging from 0 to a maximum of 0.83 (Björkman and Demmig 1987). By doing this, the outcome of these calculations can be compared with other results as long as the photosynthesis was saturated by irradiance for closing all the reaction centres.

F_m: is measured as the intensity of fluorescence when all the reaction centres are closed and photochemical quenching is zero. When all the reaction centres are closed, the photosynthesis is fully saturated and this can be accomplished by illuminating the plant by a short pulse of light of high irradiance. It was demonstrated by Schansker et al. (2011) that for dark-adapted plants, the same values for F_v/F_m were found for irradiances of the saturating light pulse between 900 and 3,000 μmol/(m^2s). During this saturating pulse of light, the fluorescence is measured by the same protocol as used for the F_o measurement. Again, the measurement of F_m itself has no direct functional trait with photosynthesis. As mentioned for F_o, F_m depends on the same instrumental layout and plant morphology. However, when F_m is measured a few seconds after the measurement of F_o with the same equipment at exactly the same position, the two images can be related on a pixel to pixel by taking the ratio.

F_v: the variable fluorescence is a measure for the maximal performance of the photosynthesis for dark-adapted plants (Butler 1978). It is the increase in fluorescence intensity from F_o to F_m. For a healthy plant with normal operating photosynthesis, the intensity of F_o can increase up to a maximum factor of 6 (Vredenberg et al. 2006). This means that $F_m = 6F_o$, and this yields for F_v/F_m the value of $(6\text{-}1)/6 = 0.83$ in agreement with Björkman and Demmig (1987). Any lower relative increase of F_v indicates that the photosynthesis is not operating at maximum efficiency. However, the value of F_v itself cannot be considered as a functional trait of photosynthesis, for the same reasons as mentioned for F_o and F_m. To use the F_v values as a functional trait of photosynthesis, the different obtained values have to be normalised by taking ratios. This is explained in the next paragraph.

F_v/F_m: for this parameter the variable fluorescence is divided by the maximum fluorescence. By performing this calculation, the instrumental geometrical parameters and the morphology of the plant mentioned before that influence the intensity of the fluorescence values of F_o and F_m that are being compensated for. As a first approximation F_v/F_m depends not on instrumental parameters like the sensitivity of the camera or the distance between the measuring beam and the plant or the distance between the camera lens and the plant nor on the amount of chlorophyll in the leaves or on the morphology of the plant but as a first approximation solely on the status of the photosynthesis expressed as a number and is an estimate for the maximum quantum yield of primary PSII photochemistry or in short the efficiency of photosynthesis (Genty et al. 1989). The intensity of the two measured fluorescence parameters can be expressed as given below (Earl and Ennahli 2004).

$$F_m = C_1 \cdot \frac{k_F}{k_F + k_D}$$

$$F_0 = C_1 \cdot \frac{k_F}{k_F + k_D + k_P}$$

With C_1 a constant that among others depends on the intensity of the excitation light, the amount of chlorophyll and σ the cross section of absorbance

and instrumental layout. The rate constant, k_F, is the rate constant for fluorescence, k_D the rate constant for thermal dissipation and k_P the rate constant for photochemistry. The maximum efficiency of photosynthesis can be derived from the ratio of F_v and F_m as given below (Butler 1978; Govindjee 1995; Earl and Ennahli 2004).

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} = \frac{C_1 \cdot \frac{k_F}{k_F + k_D} - C_1 \cdot \frac{k_F}{k_F + k_D + k_P}}{C_1 \cdot \frac{k_F}{k_F + k_D}} = \frac{\frac{k_F}{k_F + k_D} - \frac{k_F}{k_F + k_D + k_P}}{\frac{k_F}{k_F + k_D}}$$

$$= 1 - \frac{\frac{k_F}{k_F + k_D + k_P}}{\frac{k_F}{k_F + k_D}} = 1 - \frac{k_F}{k_F + k_D + k_P} \cdot \frac{k_F + k_D}{k_F}$$

$$= 1 - \frac{k_F + k_D}{k_F + k_D + k_P} = \frac{k_F + k_D + k_P}{k_F + k_D + k_P} - \frac{k_F + k_D}{k_F + k_D + k_P}$$

$$= \frac{k_F + k_D + k_P - k_F - k_D}{k_F + k_D + k_P} = \frac{k_P}{k_F + k_D + k_P}$$

This results in:

$$\frac{F_v}{F_m} = \frac{k_P}{k_F + k_D + k_P}$$

and shows that by measuring F_o and F_m, the maximum efficiency of photosynthesis can simply be calculated from only two fluorescence measured intensities. This relation is true under the assumption that the rate constants do not change when the fluorescence signals increase from F_o to F_m. F_v/F_m is an important functional trait of photosynthesis. It shows that if the maximum value for healthy dark-adapted plants is not reached, possible damage has occurred to the photosynthetic apparatus by any biotic or abiotic stress. The decrease in F_v/F_m can be a result to either a decrease in F_m or increase in F_o or both.

12.5 Light-Adapted State: Description of the Fluorescence Parameters

For plants that experience light, the situation is different from dark-adapted plants and more difficult to standardise, since the photosynthetic rate depends on many factors and one of them being the light intensity. In the light a part of the reaction centres are closed due to the absorption of light and the remaining reaction centres are open and ready to receive photons. This can be measured in the same manner as for dark-adapted plants. Genty et al. (1989) proposed the photochemical quenching and is calculated as:

$$\frac{F_q'}{F_m'} = \frac{F_m' - F_t'}{F_m'}$$

F_t': is the fluorescence intensity at time t and is measured in the same way as F_o in the dark. A prime notation used after a fluorescence parameter means that the leaf/plant is exposed to light. But as just mentioned, one has to take care of the irradiance. To be able to compare different plants, the irradiance on the leaves have to be equal. If they are different it will be difficult to compare the results. Unfortunately, F_t' has no functional trait for photosynthesis, for the same reasons as mentioned for F_o. For this reason the measurement has to be calibrated also. This is accomplished by measuring the maximum fluorescence in the light, F_m'.

F_m': the maximum fluorescence in the light-adapted state. Is it measured as the intensity of fluorescence when all the reaction centres are closed? As in the case for dark-adapted plants, when all the reaction centres are closed, the photosynthesis is fully saturated, and this is the case when the plant is illuminated by a short pulse of saturating light with high intensity. The intensity has to be higher than for dark-adapted plants (Loriaux et al. 2013). Saturating intensities can vary from species to species. It is important to ensure that saturation is achieved by measuring at increasing irradiance to test that the maximum value for F_m' has been reached.

During this saturating pulse of light, the fluorescence is measured by the same protocol as used for the F_t' measurement. Again, the measurement itself has no direct functional trait with photosynthesis, but if compared with F_m for the same plant and measuring set-up,F_m' shows a decrease compared to that of the F_m value, indicating the presence of non-photochemical quenching (see paragraph on NPQ). As mentioned before for F_t', F_m' depends on the same instrumental layout and plant morphology. However, when F_m' is measured within a second after the measurement of F_t' with the same equipment at exactly the same position, the two measurements of images can be related on a pixel-to-pixel basis and the F_m' image is used for calibration purposes.

F_q': the variable fluorescence is a measure for the effective photosynthesis for plants in the light. However, the value of F_q' itself is not a functional trait of photosynthesis, for the same reasons as mentioned for F_o and F_m.

F_q'/F_m': for this parameter the variable fluorescence is divided by the maximum fluorescence of plants at a certain irradiance. By performing this calculation the instrumental and geometrical parameters mentioned before that influence the intensity of photosynthesis are being compensated for. At a first approximation F_q'/F_m' depends not on instrumental parameters like the sensitivity of the camera or the distance between the measuring beam and the plant or the distance between the camera lens and the plant but at a first approximation solely on the status of the photosynthesis.

The status of the photosynthesis is greatly affected by the irradiance (Terashima et al. 2009). So, one has to be careful during measurements that the plant is homogeneously being illuminated. Furthermore, the irradiance under natural conditions can abruptly change and this will influence the measurements. Plants have to adapt to the given irradiance especially when they were first dark adapted. This takes normally 15–20 min (Baker and Rosenqvist 2004). F_q'/F_m' correlates with the effective efficiency of photosynthesis in the light. It is a measure how efficient the photosynthesis is operating in the light.

12.6 Non-photochemical Quenching (NPQ)

Non-photochemical quenching, NPQ, can be calculated by first measuring the F_m value for dark-adapted plants followed by F_m' for the same plant in the light: $\mathrm{NPQ} = (F_m\text{-}F_m')/F_m'$ (Bilger and Björkman 1990). The advantage of using this parameter as a measure for photochemical quenching is that it is independent of the F_o and F_t' values. NPQ can be determined in time by first measuring the F_m value for dark-adapted plants followed by measuring the F_m' value while the plant is adapting at a given irradiance (Ralph et al. 2005). Plants will be adapted after about 20 min to the irradiance and this will reach the NPQ value at the steady state. Non-photochemical quenching of chlorophyll fluorescence is an indicator of the level of non-radiative energy dissipation and provides protection from photodamage. NPQ is a measure of heat dissipation and is the total sum for the photo-protective mechanisms, state transition quenching and photo-inhibition (Krause and Weis 1991; Müller et al. 2001). Since NPQ shows increases in non-photochemical quenching in the light-adapted state compared to the non-photochemical quenching to the dark-adapted state, the calculation is only valid between plant parts having the same quenching characteristics in the dark-adapted state. This

implicates that plant parts can only be compared on their NPQ value having similar values for F_v/F_m (Baker and Rosenqvist 2004). D'Haese et al. (2004) demonstrated that NPQ can occur even at low irradiance. Stress conditions such as high irradiance or photo-inhibition and low internal CO_2 concentration due to drought or chilling (low temperature) increase NPQ. Therefore, NPQ is a functional trait of photosynthesis and serves as an indicator of stress. Imaging of NPQ has been demonstrated on sea grass by Ralph et al. (2005). An important constraint on the measurement of NPQ for imaging is that plants and leaves should not move. An NPQ image is calculated pixel by pixel from the F_m and F_m' fluorescence intensities and these pixels have to correspond to the same positions on the leaf. This implicates that imaging of NPQ is not easy since it is likely that leaves will move within a 15 min time span between the imaging of F_m and the steady state that is reached after 15 min after the light has been turned on when F_m' is being imaged. If one detects that the leaf/plant has moved, the only option is to average the F_m and F_m' values for the whole leaf/plant. This will yield averaged values for NPQ for the particular leaf/plant.

12.7 Relative Electron Transport Rate (rel-ETR)

The effective efficiency of photosynthesis, F_q'/F_m', is correlated with the photosynthetic rate. This correlation can be improved by adding two parameters, the irradiance and the coefficient of absorbance of light. A higher irradiance will in general yield a higher photosynthetic rate. A higher value for the coefficient of absorbance of light will mean that more light is being absorbed and available for photosynthesis. Taking these two parameters into account together with the effective efficiency of photosynthesis will yield a better trait of photosynthesis. This can be expressed in an equation for the relative electron transport rate. It is important to establish this relation between the electron transport rate and the measured fluorescence parameters. Walker et al. (1983) found an inverse relation between oxygen evolution and simultaneously measured fluorescence: oxygen and fluorescence displayed marked dampening oscillations that were anti-parallel, meaning almost 180° was out of phase. They further showed that CO_2 uptake behaved like oxygen evolution. These results indicated that fluorescence measurements can be used to be correlated with the photosynthetic rate. Genty et al. (1989) found a linear behaviour between the uptake of number (#) of CO_2 molecules per number of absorbed photons and the effective efficiency of photosynthesis for mature maize leaves. This can be written as:

$$C_2 \frac{\#CO_2}{\#photons} = \frac{F_m' - F_t}{F_m'}$$

This empirical equation demonstrates that a simple relation exists. Seaton and Walker (1990) showed that this relation was valid for 16 different species with a constant $C_2 = 8$. Edwards and Baker (1993) found for C_2 a value between 11 and 13 for maize leaves. This is a different value as Seaton and Walker (1990) observed, but they did not correct for the absorbance of light. Fryer et al. (1998) confirmed the relation for maize leaves and demonstrated that a value of $C_2 = 12$ fitted this relation well. This means that it takes 12 electrons for the uptake of one CO_2 molecule, six from PSII and six from PSI. It is assumed that for both photosystems, this value is equal. An equation for the relative electron transport rate can be derived now from the empirical relation:

$$12 \frac{\#CO_2}{\#photons} = C_3 \frac{\#CO_2}{PPFD * \alpha_L} = \frac{F_m' - F_t}{F_m'}$$

where the number of photons has been replaced by PPFD * α_L with PPFD the photosynthetic photon flux density, or irradiance, in μmol quanta of photons per m^2 per s and α_L the absorption coefficient of the leaf. Since the number of CO_2

molecules directly correlates with the relative ETR, one can derive using $C_2 * \#CO_2 =$ rel-ETR:

$$\text{rel-ETR} = \frac{F_m' - F_t}{F_m'} * \text{PPFD} * \alpha_L * \frac{\text{PSII}}{\text{PSI}}$$

First, the relative electron transport rate, rel-ETR, depends on the effective efficiency of photosynthesis calculated from the fluorescence signals and expressed as $(F_m'-F_t')/F_m'$, the intensity of the irradiance capable of inducing photosynthesis, called photosynthetic photon flux density, PPFD, the absorption coefficient, α_L, and PSII/PSI = proportion of light absorption by PSII and PSI (Baker et al. 2007). The application of the calculation of an image of rel-ETR has been demonstrated for sea grass by Ralph et al. (2005).

12.8 Fluorescence Depends on Wavelength, Plant Species and Cultivar

For healthy dark-adapted plants, one can find in literature that the value of $F_v/F_m = (F_m\text{-}F_o)/F_m$ has a maximum value of 0.83 (Björkman and Demmig 1987). These values can be obtained using plants that are grown at optimal conditions, not suffering or subjected to any stress and well dark adapted. Practical values found in literature are lower in value, around 0.77 (Jansen et al. 2009). However, great care has to be taken about the excitation wavelengths that are being used in the measuring protocol. There exists a slight dependence of F_v/F_m on the excitation wavelengths for inducing the chlorophyll fluorescence (Pfündel 2009; Hogewoning et al. 2012). Not only F_v/F_m depends on the excitation wavelength but on the fluorescence wavelength also. This is due to the spectral dependence of the fluorescence on the two photosystems. Around 680 nm the fluorescence is largely associated with PSII. At increasing wavelengths fluorescence is also emitted by PSI superimposed on the PSII fluorescence (Franck et al. 2002). This results in lower F_v/F_m values for measurements which take higher wavelengths into account. Differences in F_v/F_m were also observed depending on the species and even on the phenotype (Rock et al. 1992; Pfündel 2009).

12.9 Examples of Using Chlorophyll *a* Fluorescence in Phenotyping

Chlorophyll fluorescence analysis is a non-destructive technique. This has been used successfully to assess the resistance against biotic and abiotic stresses in crop plants. Thus phenotyping of traits related to photosynthesis on the basis of the above technique has been used to characterise the genotypes as resistant/tolerant and sensitive/susceptible to pathogen, heat, drought, etc. For example, heat stress is an increasing constraint for the productivity of wheat and also other crops due to the global climate change. In wheat, a chlorophyll fluorescence protocol was standardised and used for mass screening of 1,274 wheat cultivars of diverse origin. The maximum quantum efficiency of PSII (F_v/F_m) was used to screen the heat-tolerant genotypes. The initial mass screening of the above genotypes with a milder heat stress of 38 °C in 300 μmol m^{-2} s^{-1} for 2 h with preheating at 33−35 °C for 19 h in 7−14 μmol m^{-2} s^{-1} light showed a genetic determination of 8.5 ± 2.7 % and led to identification of 138 genotypes. Further, these selected genotypes have screened under a heat treatment of 40 °C in 300 μmol m^{-2} s^{-1} for 72 h and resulted in larger differentiation of cultivars with an increased genetic component (15.4 ± 3.6 %). Finally it was further increased to 27.9 ± 6.8 % in the third screening with 41 contrasting cultivars. After this, the contrasting set of genotypes has been compared on the basis of chlorophyll fluorescence parameters in order to detect genetic difference for heat tolerance. This has resulted in the identification of a set of wheat genotypes having contrast for their inherent photochemical efficiency. This may aid future studies to understand the genetic and physiological nature of heat stress tolerance in order to dissect quantitative

traits into simpler genetic factors (Sharma et al. 2012).

Similarly the maximum quantum yield of photosystem II (PSII) photochemistry ($F_v/F_m = (F_m\text{-}F_0)/F_m$) has also been shown interesting for phenotyping disease severity. The ratio of F_v/F_m has been used to display a robust contrast between infected and healthy tissues. Generally healthy tissues had F_v/F_m values around 0.84 in several plant species, while susceptible genotypes showed this value lower than this. In many studies, mean F_v/F_m measurements were used to qualitatively discriminate between diseased and healthy leaves (see Rousseau et al. 2013 and references therein for more details).

12.10 New Developments and Future Perspective

On the instrumentation level, new developments are being employed in the direction of measuring a better estimate for the F_m' value. It was, several decades ago, already observed that the measurement of F_m' did not saturate the photosynthesis. Not all reaction centres could be closed. Increasing the irradiance yielded still an increase in the fluorescence intensity (Markgraf and Berry 1990). Using higher irradiance for saturating the photosynthesis would involve damage to the photosynthetic system due to photo-inhibition (Müller et al. 2001). One way to overcome this dilemma is by measuring the F_m' intensities at different irradiances. Markgraf and Berry (1990) and Earl and Ennahli (2004) demonstrated that a linear dependence exists between the fluorescence intensity and the inverse of the irradiance, $(PPFD)^{-1}$. Using this linear dependence and linearly extrapolating to infinite irradiance, a better estimate was calculated for F_m'. However, the measurements at different irradiances cannot be performed at high rate, because between each measurement at different irradiance, there was a time delay of several minutes. This drawback was overcome by Loriaux et al. (2013) by development of a novel saturation-pulse method referred to as a multiphase flash (MPF). Within one flash of less than 1 s, a variation of irradiances is included. Simultaneously the fluorescence is measured at each irradiance. This yields rapidly the dependence of F_m' at different irradiances and the estimation of a better F_m' at infinite irradiance. This method now exists for spot measurements, but the challenge is to apply this method for imaging also.

Finally, all this effort on the instrumental level is done to provide tools to characterise the status of the photosynthesis. This enables selection of cultivars or genotypes with the best performance at given environmental factors. Another possibility to increase crop production is by changing the mechanism of photosynthesis (Murchie et al. 2008; Murchie and Niyogi 2011). Several routes are being mentioned like improvement of CO_2 assimilation rates, decreasing photorespiration and transforming C_3 into C_4 crops.

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Identification of Subcellular, Structural, and Metabolic Changes Through NMR

13

Rekha Sapru Dhar and Nupur Malviya

Abstract

Secondary metabolites are unique sources for flavors, nutraceuticals, pharmaceuticals, and industrial bioactive molecules which are biosynthesized in different plant tissues. These metabolites play a major role in the adaptation of plants to the prevailing environment, in overcoming stress conditions, and in defense to several unforeseen invasions. Identifying the biological components and their functions and multiple interactions between components to understand the cellular metabolism of a cell to meet its fluctuating demand for energy and materials has remained a challenging task. Based on traditional metabolic analysis, mapping of intracellular fluxes in metabolic networks is only possible with high-throughput techniques. Nuclear magnetic resonance (NMR) spectroscopy is a powerful and versatile tool that can provide information on the metabolites and their metabolic network. NMR has played a dominant role in the identification of an array of compounds from diverse environments and diverse ecogeographic domains. Plant biotic relationships which include host plant interaction and resistance for an eco-metabolomics have been developed with NMR approach. NMR can also be used to determine low-resolution structures of target–ligand complexes for natively unstructured proteins or membrane proteins that are not amenable to crystallographic approaches.

Keywords

Metabolites • Elicitors • Profiling • Crop plants

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13.1 Introduction

During the process of evolution, plants have produced a vast and rich chemical diversity. Several other processes such as mutation and gene duplication have also led to the formation of new

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chemical structures to provide adaptability to plants in different environments. There are almost 200,000 known secondary metabolites with different chemical structures, belonging to different classifications with different functions, which include lipids, sugars, oligonucleotides, peptides, nucleosides, ketones, amines, etc. Exposure of plants to various biotic and abiotic stresses has significantly changed the synthesis of secondary metabolites and their extensive subcellular compartmentalizations. Among environmental factors, light, water, and salinity are important variables affecting phytochemical production in plants (Perez-Balibrea et al. 2008). For example, when plants are exposed to drought stress, they exhibit a wide range of responses both at cellular and molecular levels (Chaves et al. 2003). Identification of a metabolic network helps to know the pathway for sustaining the plants in diverse environmental conditions. Therefore, the focus has been on the high-throughput profiling of metabolite contents as a trait to screen genetic resources. There are several examples of metabolo-phenotype-based breeding programmes including carotenoid content in tomato (Liu et al. 2003), protein and oil content in maize (Moose et al. 2004), and starch content in rice and potato (Ferine and Willmitzer 2004). Metabolite profiling has also been applied to identify Quantitative trait loci (QTLs) in association with metabolite accumulation to dissect the genetic basis of metabolic network in *Arabidopsis*, tomato, and poplar (Kliebenstein et al. 2001, 2002; Tieman et al. 2006). In future integrative analysis of metabolomics and genomics or transcriptomics should be facilitated to elucidate plant metabolic systems as well as to explore key loci applicable for crop improvement. Therefore, studying the metabolic phenotype is important to understand the genetic changes in crop plants towards the tolerance or resistance against both biotic and abiotic stresses. An advanced and efficient tools have been developed in phenomics of plant metabolites for detecting the changes undergoing in the plant system and identifying the already existing molecules or some novel analogues with new functions. Monitoring of metabolites helps to speed up the discovery of drugs, their development and identification of new functions of genes in order to understand the biology of plants with the knowledge gained by genomics, proteomics, transcriptomics, metabolomics, and the nutritional values of plants for health care. NMR is a tool that can provide detection and survey of metabolites along with their associated subcellular and cellular energetic and metabolic fluxes under normal and stress conditions. Therefore, in this chapter, the role of NMR technique has been discussed in the identification of subcellular, structural, and metabolic changes in crop plants. This is a nondestructive technique, which can be used in combination with other complementary techniques, like GC-MS and liquid chromatography.

13.2 Principal of NMR

Resonances of magnetic nuclei such as 1H, 13C, and 15N that interact with an external magnetic field can be measured by NMR spectroscopy. NMR allows in vivo analysis of metabolites in crude extracts, cell suspensions, intact tissues, or whole organisms (Fan and Lane 2008). For each single compound, NMR spectra are unique and specific (Verpoorte et al. 2007, 2008) and can be used to identify metabolites of biological origin without prior knowledge (Fan and Lane 2008). Metabolites which are primarily composed of carbon, nitrogen, hydrogen, oxygen, phosphorous, and sulfur have magnetic isotopes which are detectable by NMR. It identifies and quantifies metabolites when a sample is placed in the magnet of NMR spectrometer. The principal of NMR is based on the fact that nuclei of atoms have magnetic properties that can be utilized to yield chemical information. Protons, neutrons, and electrons (quantum subatomic particles) have spins. These spins are paired in some atoms (e.g., 12C, 16O, 32S) and cancel each other out so that the nucleus of the atom has no overall spin. However, the nucleus does possess an overall spin in many atoms (1H, 13C, 31P, 15N, 19F, etc.,). There are rules for the determination of a spin of a given nucleus, for

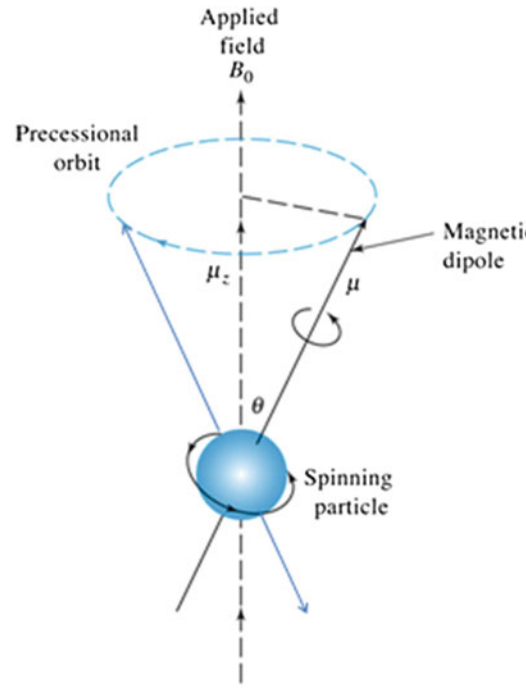
example if the number of neutrons and the number of protons are both even, the nucleus has no spin and if the number of neutrons plus the number of protons is odd, then the nucleus has a half-integer spin (i.e., 1/2, 3/2, 5/2). Similarly, if the number of neutrons and the number of protons are both odd, then the nucleus has an integer spin (i.e., 1, 2, 3).

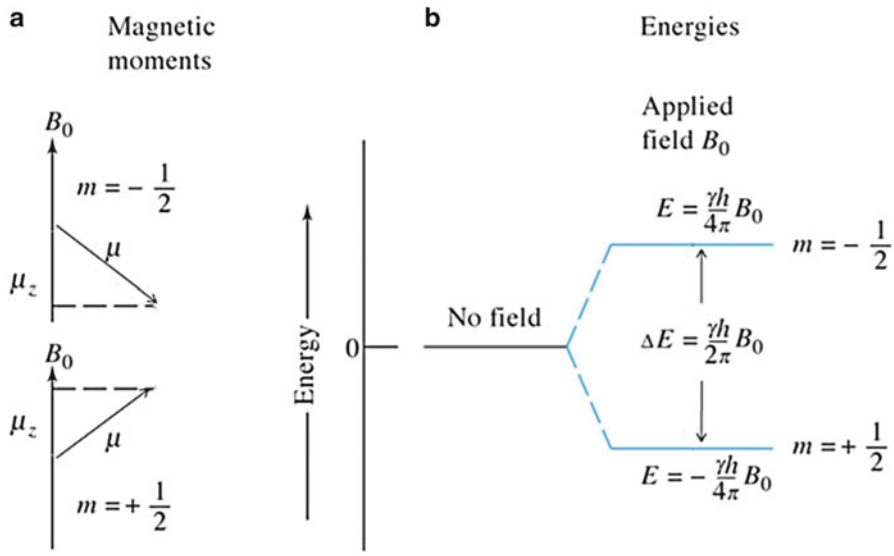
The resulting magnetic moment μ is oriented along the axis of a spin and is proportional to the angular momentum p. Thus,

$$\mu = \gamma p$$

where the proportionality constant γ is the magnetogyric ratio, which has a different value for each type of nucleus. In quantum mechanical terms, when a nucleus with a spin quantum number of one-half is brought into an external magnetic field B_0, its magnetic moment becomes oriented in one of two directions, one is $-1/2$ and another is $+1/2$, with respect to the field depending upon its magnetic quantum state.

The magnetic moment of the lower energy $+1/2$ state is aligned with the external field, and that of the higher energy $-1/2$ spin state is opposed to the external field.





Difference in energy between the two states is given by

$$\mathrm{DE} = ghB_0/2p$$

where:
B_0 – external magnetic field
h – Planck's constant
g – magnetogyric ratio

When the energy of the photon matches the energy difference between the two spin states, absorption of energy occurs, that phenomenon is known as resonance:

$$\mathrm{DE} = hu = ghB_0/2p \quad \text{So,} \quad u = gB_0/2p.$$

When radio frequency energy matches the Larmor frequency at a right angle to the external field, it would cause a transition between the two energy levels of the spin, and the precessing nucleus will absorb energy and the magnetic moment will flip to its $I = -1/2$ state.

There are several types of NMR spectra, which are dependent on the kind of instrument used, the type of nucleus involved, the environment of the analytic nucleus, the physical state of the sample, and the data collection. Mostly, wide line or high resolution constitutes the NMR spectra.

High-resolution spectra are collected by the instruments which have the capability of differentiating between very small frequency differences of 0.01 ppm or less and can resolve two peaks into additional peaks. In the lower-resolution spectrum for the protons in ethanol, three peaks are observed arising from absorption by the CH_3, CH_2, and OH protons. But in the higher-resolution spectrum, two of the three peaks can be resolved into additional peaks. There are two main environmental effects on NMR spectra, chemical shift and spin–spin splitting.

The quality of an NMR analysis is not dependent on the number of signals detected but on the number of metabolites identified. The NMR method provides simultaneous access to both qualitative and quantitative information since the signal intensity is directly proportional to the molar concentration. NMR has different facets like microscopy, spectroscopy, and imaging that allow generating fingerprints with high qualitative estimation and reproducibility with their 1D, 2D, and 3D structures and also allow visualizing the intact tissues along with their functions. Several imaging techniques are being utilized to detect the early signs due to stress by monitoring the changes in photosynthetic efficiencies, structural modifications, status of water, and accumulation of secondary metabolites. Correlated spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC) spectroscopy, heteronuclear single-quantum coherence (HSQC) spectroscopy, and heteronuclear multiple-bond coherence (HMBC) spectroscopy are also being exploited for improved metabolite identification by providing information on the relationship between the signals from two different nuclei (Ratcliffe et al. 2001).

13.3 Role of NMR in Plant Metabolite Profiling

Plant metabolism is more complex as compared to microorganisms, because of the existence of several subcellular compartments and metabolic fluxes mediated by the pathways involved in the biosynthesis of a plethora of metabolites of diverse types. There are several factors including genetic modifications physiological, pathophysiological, and developmental stimuli which are responsible to make changes in metabolic profiling (metabolomics/metabonomics). The environmental conditions such as flooding, freezing, drought, salinity, and temperature ultimately affect the metabolism of the plant to yield various metabolites which provide adaptability to plants against these stresses and a defensive force against pathogens or insect invasions. NMR plays a significant role to investigate the impact of environmental factors (i.e., growth conditions, exposure to metals, herbicides, and cultural practice) on metabolic changes in plants. It has also been applied to study the difference in metabolites between developmental stages and hybridized plants and also to characterize several ecotypes with resistant property against stresses

Table 13.1 NMR used for metabolomic studies in different plant species

Target	Species	References
Fungal induced	*Brassica rapa*	Abdel-Farid et al. (2009)
Metabolomic fingerprinting	*Grapevine*	Ali et al. (2009)
Host plant interactions		Allwood et al. (2008)
Metabolic profiling		Avelange-Macherel et al. (2006)
Cadmium effect	*Silene cucubalus*	Bailey et al. (2003)
Changes in ferulic acids	Barley	Cabrera et al. (1995)
Drought stress	*Pisum sativum*	Charlton et al. (2008)
Metabolic fingerprinting	*Tabaccum*	Choi et al. (2004a)
Matabolic differentiation	*Cannabis sativus*	Choi et al. (2004b)
Metabolic discrimination	*Catharanthus roseus*	Choi et al. (2004c)
Metabolic fingerprinting	*Ilex* spp.	Choi et al. (2005)
Metabolic profile	Tobacco mosaic virus	Choi et al. (2006)
Fungal pathogens	Bromegrass	Delgado (2002)
Fungal pathogens	*Vitis* spp.	Figueiredo et al. (2008)
Elicitors	*Manihot esculenta*	Gomez-Vasques et al. (2004)
Metal ion induced	*Brassica rapa*	Jahangir et al. (2008a, b)
Plant hybrids		Kirk et al. (2005)
Transformation	*Lycopersicum esculantum*	Le Gall et al. (2003)
Caesium effect	*Arabidopsis thaliana*	Le Lay et al. (2006)

(see review Leiss et al. 2011). Its use in the identification of subcellular, structural, and metabolic changes has been reviewed earlier (Roberts 2000; Köckenberger 2001a, b; Bligny and Douce 2001; Pfeffer et al. 2001; Ratcliffe and Shachar-Hill 2001; Ratcliffe et al. 2001; Keifer 1999; Hemminga and Visser 2000; Lens and Hemminga 2001). NMR-based techniques have been widely used either for the quantification of the most abundant compounds or single class of compounds. However, it has also been used in the quantitative measurement of metabolites in crop plants. Table 13.1 lists the studies in which NMR was used to identify the subcellular compounds and metabolic changes occurred during stress of different kinds and stimuli in the following text.

13.3.1 Temperature

Plants encounter different kinds of temperatures from low to high during their life span. Plant makes several metabolic changes for survival against these variable temperature regimes. The freezing temperature causes dehydration and cell deformation in plants. The water content present in plant starts ice formation. As a result, plant develops a mechanism that overcomes or adapt to that conditions. Different tissues of plants respond differently to such harsh stimuli. For example, bark undergoes extracellular freezing and parenchyma cells of xylem rays show super-deep cooling, whereas the flower buds and leaves undergo extra-organ freezing. Using NMR microscopy, all these characteristics can be visualized and can be analyzed in detail in order to understand the responsible regulatory mechanisms in different plant species. At decreasing temperature, intensity of NMR signals also decreases leading to the identification of intracellular freezing in the plant tissues. In *Brassica*, NMR was used to analyze the structure of low temperature-induced protein (Boothe et al. 1997). NMR spectroscopy quantifies liquid water. However, a simple NMR cannot show the location of ice and water formation, but NMR microimaging (magnetic resonance imaging, MRI) has sufficient resolution to identify supercooled water in small organs or in tissues of the dimension of xylem (Ishikawa et al. 1997). Endemic seaweeds in the genus *Porphyra* are

widely cultivated on a commercial scale worldwide. Two colored mutants of *Porphyra haitanensis*, namely, Shengfu 1(SF-1) and Shengfu 2(SF-2), have shown to have good high-temperature tolerance. NMR methods have also been used to understand the molecular aspects of high-temperature tolerance by studying the metabolic differences between the high-temperature-tolerant strains and wild type. It has been observed that the high-temperature-tolerant strains have significantly different metabolic phenotypes from the wild type and have significantly higher levels of a set of osmolytes consisting of betaine, taurine, laminitol, and isofloridoside than the wild type, indicating the particular importance of efficient osmoregulation for high-temperature resistance. Thus, NMR-based metabolomics has been observed as a useful tool for understanding the metabolic features related to resistance to temperature stresses (Ye et al. 2013).

13.3.2 Drought and Salinity

Drought stress induces a series of morphological, biochemical, and physiological changes and affects plant adversely for its growth and productivity. It often causes oxidative stress and shows an increase in the amounts of flavonoids and phenolic acids in the leaves of some plants. Sharma et al. (2012) identified low levels of total alkaloids, flavonoids, and tannins when plants were grown under drought and salinity conditions. Similar results were also reported by Murch et al. (2003) when plants of *Hypericum perforatum* were exposed to nickel. In soybean, ^{1}H NMR-based metabolite analysis combined with the physiological studies was used to detect metabolic changes in drought-tolerant (NA5009RG) and drought-sensitive (DM50048) genotypes in order to elucidate metabolic adjustments in relation to the physiological responses in the nitrogen-fixing plants towards water limitation. The results of this analysis demonstrated critical differences in physiological responses between these two genotypes and identified the metabolic pathways that were affected by short-term water limitations in soybean plants. The study also highlighted pools of metabolites that play a role in the adjustment of metabolism and physiology of soybean varieties to meet drought effects (Silvente et al. 2012).

Drought and salinity increase saponin content in plants (Hernandez et al. 2000). This observation was in support with the results reported by De Costa et al. (2013) that saponin content in *Quillaja brasiliensis* leaves increased significantly when exposed to salinity. El-Sayed et al. (2008) also reported that saponin content in *Tribulus* increased when subjected to water stress. This increase was related to its protective role against oxidative stress (Lin et al. 2009). Quantitative analysis of *Acalypha wilkesiana* showed that plants under drought and salinity stresses produced low quantity of alkaloids, flavonoids, and tannins whereas saponin production was increased, such negative influence of salinity on plants via photosynthesis inhibition, ion toxicity, and water deficit was demonstrated (Odjegba and Alokolaro 2013). The content of saponins in *Chenopodium quinoa* from 0.46 % dry weight (dw) in plants growing under low water deficit conditions to 0.38 % in high water deficit plants was changed. Flavonoids have protective functions during drought stress. The physiological response to drought or salt stress is due to the role of membrane permeability especially during drought stress, whether to facilitate the water transport for the expansion or growth of the plant during stress or to decrease the permeability for preserving cellular water within tissue (Aubert et al. 1999). It has been reported that membrane permeability is regulated by the expression of aquaporin genes or water channels, either the aquaporin genes upregulated or downregulated the permeability of membranes and hydraulic conductance during drought. Membrane permeability is frequently determined using the NMR relaxation times of intracellular water protons (Snaar and Van As 1992; Zhang and Jones 1996; van der Weerd et al. 2002).

13.3.3 Metal Toxicity

NMR is useful to identify the causes of detoxification in plant cells. The resistance for aluminum (Al) in signal grass and less-resistant ruzi grass could be due to the accumulation of Al in roots (Wenzl et al. 2002). It has been shown that approximately two-third of the total Al makes a complex with soluble low molecular weight ligands, which are taken up into the root symplasm. Later on, this conclusion was confirmed in another study by a ^{27}Al NMR analysis of *Brachiaria* hybrid cv. *Mulato*, which showed that Al in the root symplasm was present as a complex with ligand(s) (Watanabe et al. 2006). Possible candidates for such ligands include citric acid, malic acid, *trans*-aconitic acid, oxalic acid, and 1,3-di-*O*-*trans*-feruloylquinic acid, a chlorogenic acid analogue previously isolated from Brachiaria grass roots (Wenzl et al. 2000). These ligands may constitute a sink for Al ions in matured roots because very little Al is translocated to shoots (Wenzl et al. 2002).

13.3.4 Biotic Stresses

The metabolic profiles in reference to defense-related secondary metabolism have been detected in several studies. Carotenoid profiling (Frasier et al. 2007), phenylpropanoid and monoterpenoid indole alkaloid biosynthesis in phytoplasma-infected*Catharanthus roseus* leaves (Choi et al. 2004a, b, c), and hydroxycinnamates and glucosinolates accumulation in methyl jasmonate (MeJA)-treated *Brassica rapa* leaves (Liang et al. 2006) are some examples. Although the use of 1H NMR for metabolite fingerprinting in the biomedical field is well established and extensive, than plants (Ward et al. 2001). Leiss et al. (2009a, b) identified the candidate compounds for the constitutive host plant resistance to western flower thrips (*Frankliniella occidentalis*) on the basis of NMR in *Senecio* as a wild plant, chrysanthemum as an ornamental, and tomato as a crop. They selected thrips-resistant and thrips-susceptible plants for NMR metabolomics and identified a range of different metabolites involved in thrips resistance. In the wild plant *Senecio*, the second generation hybrids of *S. jacobaeae* and *S. aquaticus* resistant to thrips contained significantly high amount of pyrrolizidine alkaloids (PAs), jacobine- and jaconine N-oxide, and a flavonoid, kaempferol glucoside. Similarly in ornamental chrysanthemum, thrips-resistant cultivars showed significantly high amount of phenylpropanoids, chlorogenic and feruloyl quinic acids. In tomato, wild species resistant to thrips contained acylsugars, which are known for their negative effect on caterpillars, leaf miners, whiteflies, and aphids, compared to chrysanthemum, tomato contained relatively small amount of chlorogenic acid (Mirnezhad et al. 2010).

The use of NMR in studying the metabolome of the infected and noninfected hosts of *Catharanthus roseus*, *Spiroplasma citri*, and *Arabidopsis thaliana* has been reviewed earlier in detail (Leiss et al. 2011). Relatively, little attention has been given to the use of NMR to study constitutive host plant resistance to pathogens, though two studies have been reported in grapes. In the first study, cultivars resistant to mildews accumulated inositol and caffeic acid. These compounds have reported to confer resistance to fungi (Figueiredo et al. 2008). In another study, differentiation of grape cultivars based on their resistance to downy mildew was based on quercetin-3-*o*-glucoside and a *trans*-feruloyl derivative (Ali et al. 2009).

13.3.5 Elicitation

Plant cell cultures have the capability to produce an array of secondary compounds. Changes in secondary metabolites of plants due to the intervention of environmental perturbation are associated with a number of factors, out of which elicitations with chemicals or fungi are also the key components. Elicitations can be biotic, which involves plant-derived compounds or microorganisms derived (Keen et al. 1972) compounds, or abiotic, which involves physical or chemical stress factors affecting the production of metabolites (Davis et al. 1986). Proton nuclear magnetic resonance (1H NMR) can be used to investigate the interplay between two metabolisms, primary and secondary,

when the cultures of plants are elicited with different chemicals and fungi. Several elicitors are being employed in *in vitro* cultures of plants for the increased production or induced *de novo* synthesis of secondary metabolites (Sudha and Ravishankar 2003a; Karuppusamy 2009). For example, 2,4-dichlorophenoxy acetic acid (2,4-D), indole acetic acid (IAA), and naphthalene acetic acid (NAA) have increased the production of indole alkaloids in cell cultures of *Cinchona ledgeriana* (Harkes et al. 1985). Half-strength Gamborg's B_5 medium supplemented with 5 % sucrose and 1 mg/l of each of NAA and BA gave the best values in root culture growth of *Datura candida* x *D. aurea* (Nussbaumer et al. 1998), whereas full-strength B_5 medium supplemented with the same concentrations of NAA and BA gave the best results for hyoscyamine and scopolamine content.

Similarly various signaling molecules like methyl jasmonate (MeJA), jasmonic acid (JA), or salicyclic acid (SA) in both biotic and abiotic stresses act as elicitors in a wide spectrum of signaling pathways and produce several compounds (alkaloids, phenolic phytoalexins, taxanes, coumarins, etc.). Both molecules (MeJa and SA) exogenously supplied in the cultures have affected the morphology, physiology, and secondary metabolism of the plants. For example, MeJA increased the shikonin and its derivatives (red naphthoquinones) in *Onosma paniculatum* cultured cells (van der Fits and Memelink 2000). Exogenously applied MeJA has increased anthocyanin in *Arabidopsis thaliana* (Perez et al. 1997) and putrescine and spermidine in buckwheat (Horbowicz et al. 2010), whereas SA induced anthocyanin in *D. carota* cultures (Sudha and Ravishankar 2003b).

13.3.6 Quantitative Analysis of Metabolites

Metabolic profiling is becoming a quite useful technology for microscopic and comprehensive phenotyping and diagnostic analysis in plants and as a key approach to annotate gene function and systematic evaluation of metabolites (Schauer and Fernie 2006). Metabolic phenotyping is applicable to the holistic discovery of metabolite markers as well as nutrition-targeted breeding programmes based on high-throughput profiling of metabolite contents as a trait to screen genetic resources. There are now several examples of metabolophenotype-based breeding that include carotenoid content in tomato (Liu et al. 2003), protein and oil content in maize (Moose et al. 2004), starch content in rice and potato (Fernie and Willmitzer 2004). NMR has been used widely to select phenotypes on the basis of oil content in various plant species. In castor bean, a collection of 1,033 accessions from 48 countries were screened for oil content. This analysis revealed an average of 48.2 % oil with a range from 37.2 to 60.6 % (Wang et al. 2011). Similarly, phenotyping of oil content has been done in *Lesquerella fendleri* which led to the development of a new crop for arid regions of the Southwestern United States as an alternative source of hydroxy fatty acids (HFAs). In this study, 66 accessions from 28 species of *Lesquerella* collected from the United States, 33 accessions from 4 species of *Lesquerella* from Mexico, and 41 accessions from 15 species of *Physaria* were analyzed for seed oil content. It ranged from 32.2 % in *Lesquerella* to 35.4 % in *Physaria* (Salywon et al. 2005). Metabolic profiling was applied to identify QTLs (Quantitative trait loci) in association with metabolite accumulations to dissect the genetic basis of metabolic network in *Arabidopsis*, tomato, and poplar (Kliebenstein et al. 2001, 2002; Tieman et al. 2006). In future, integrative analysis of metabolomics and genomics or transcriptomics should be facilitated to elucidate plant metabolic systems as well as to explore key loci applicable for crop improvement.

13.4 Conclusions

The increased demand of agriculture as well as phytoceuticals globally has placed numerous investigating tools and has pressurized to develop more sophisticated and accurate machinery to overcome limitations. The contribution of

NMR towards the understanding of plant protective tissues at molecular level from different sources like water and solute loss, pathogen invasions, and UV irradiation has been well illustrated. Therefore, NMR has proved and would prove to be a potential means to provide useful information with respect to essential phytochemicals vis-à-vis subcellular complementation for future benefits to mankind by developing targeted drugs, except for its limited information regarding the molecular structures of plant polymers due to their heterogeneity. Though, challenges like sensitivity and spectral resolution were tried to overcome by using modified, direct, and cross-polymerization experiments. However, challenges remained as such due to the different swelling capabilities of different components resulting in low representation of densely cross-linked moieties because of their insufficient exposure to solvent.

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Precision Nutrient Management and Crop Sensing

14

Jerry L. Hatfield

Abstract

Sensing of nutrient status in crop plants is achievable with remote sensing techniques because the nutrient concentration affects the reflectance spectrum. Techniques have been developed with both active and passive sensors engineered to detect the reflectance in specific wavebands and applied mainly to nitrogen status in maize and wheat canopies based on the observation that changes in spectral indices are correlated with plant biomass in the early stages of plant development, and if these deficiencies were due to nitrogen, then additions of nitrogen would allow the plant to achieve potential yield, if there is no other limitation to production, e.g., water or pests. There has been extensive research on the use of techniques which mainly use the normalized difference vegetative index (NDVI); however, the management tools rely on the use of a nitrogen-rich strip in the field. The positive aspects of improving nutrient management are the potential for improved precision management both spatially and temporally. Although, the current approaches have been evaluated for a number of crops in addition to maize and wheat, there remain some challenges in application of the methods which may potentially be overcome by evaluating other spectral methods which are more sensitive to canopy chlorophyll content and less sensitive to biomass. Application of technologies to improve nitrogen management has been shown to have a positive impact on reducing nitrogen application, improving yield of grain and sugar in sugar beets and sugarcane, increasing profitability, and decreasing the negative effect from excess nitrogen in the environment.

14.1 Introduction

Improved nutrient management will lead to enhanced nutrient use efficiency and reduced environmental impacts from agricultural systems. However, the avenues to achieve both

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improved nutrient use efficiency and environmental quality have proven elusive because of the inability to precisely predict the nutrient needs of the crop to ensure no major excesses in nutrients exist at the end of the growing season. Estimates of plant nutrient availability from soil tests have not always proven reliable, and plant tissue tests require destructive sampling of the plant, and subsequent chemical analysis before an assessment can be made of the nutrients required by the plant. The development of techniques which can nondestructively estimate the nutrient status in a plant canopy rather than a single leaf is considered to be a potential answer to improved nutrient management. Over the past two decades, there have been advances in the use of various remote sensing methods to estimate nutrient status in crops and provide recommendations on the amount of nutrient required to reduce the nutrient stress, if it is present, and maximize crop productivity. These techniques offer promise for improved nutrient management of different crops. This chapter presents an overview of the current status of crop sensing and the results obtained across a number of crops. To fully understand and appreciate the potential of crop sensing and its application to nutrient management, it is important to present a background on the changes of crop reflectance during the growing season and how these relate to different vegetative indices and finally how these are applied to nutrient sensing.

14.2 Crop Reflectance Patterns During a Growing Season

Optical properties of plants have been studied for a long time, and a recent review by Hatfield et al. (2008) describes the early work of the remote sensing pioneers that has provided the basis for our current use of crop reflectance as a management tool. The development of various vegetative indices based on the reflectance of leaves and canopies utilizes the observation that there is a changing reflectance for different wavelengths throughout the year as plants grow and develop. Vegetative indices in the generic sense are combinations of wavebands in some form which have been related to a crop parameter ranging from simple ratios of wavebands, e.g., R_{NIR}/R_{red} to more complex models like the soil-adjusted vegetative index (SAVI) expressed as $\text{SAVI} = (R_{NIR}\text{-}R_{red})(1+L)/(R_{NIR}+R_{red}+L)$. The change in the reflectance pattern for a canopy throughout the growing season is necessary for the vegetative indices to have value in assessing a change in crop growth. The different combinations of wavebands and the crop parameter best represented by this index are shown in Table 14.1. Hatfield and Prueger (2010) showed the temporal distributions of many different vegetative indices across a growing season to demonstrate the utility of these indices in following the temporal patterns of plant canopy development.

The reflectance surface for a maize (*Zea mays* L.) canopy from 0.5 to 1.7 μm throughout a growing season provides a view of the changes which occur among the wavelengths and how different vegetative indices would potentially provide a relationship to crop growth and development (Fig. 14.1). The magnitude of the change in the reflectance of the visible wavelengths (0.5–0.7 μm) is not as large as the change in the near-infrared (0.7–1.1 μm) wavelengths, while the shortwave infrared (1.1–1.7 μm) wavebands show less change throughout the season; however, these changes are all different compared to the soil background (Fig. 14.1). Therefore, as a canopy develops, there is a decrease in the red reflectance because the leaves become more effective at capturing light as the number of total leaves present increases because the reflectance of an individual leaf does not change, but the presence of more leaves in the canopy increases the effectiveness in absorbing incoming solar radiation. Conversely, the reflectance in the near-infrared wavebands increases as the canopy develops because the overall structure of the canopy increases the near-infrared reflectance (Fig. 14.1). These changes show why simple ratios can be related to accumulation of biomass by the crop during the growing season. The soil background line is represented by a linear relationship and does not exhibit the changes in reflectance in individual wavebands which allows for the discrimination of plants from soils (Baret et al. 1989).

Table 14.1 Summary of selected vegetation indices, wavebands, and applications

Index	Wavebands/formula	Application	Reference
	$R_{800} - R_{680}$	Biomass	Jordan (1969)
	$R_{800} - R_{550}$	Biomass	Buschmann and Nagel (1993)
	R_{550}	Chlorophyll	Carter (1994)
	R_{700}^{-1}		Gitelson et al. (1999)
	$\log(1/R_{737})$	Chlorophyll	Yoder and Pettigrew-Crosby (1995)
Simple ratio	$R = R_{\text{NIR}}/R_{\text{red}}$	Biomass, LAI, cover	Birth and McVey (1968), Jordan (1969), Tucker 1979
Photochemical reflectance index	$\text{PRI} = (R_{550} - R_{531})/(R_{550} + R_{531})$	Light capture efficiency	Gamon et al. (1992)
Pigment-specific normalized difference	$(R_{800} - R_{470})/(R_{800} + R_{470})$	Leaf area index	Blackburn (1998)
Normalized difference vegetation index	$\text{NDVI} = (R_{\text{NIR}} - R_{\text{red}})/(R_{\text{NIR}} + R_{\text{red}})$	Intercepted PAR, cover	Deering (1978)
Perpendicular vegetative index	$\text{PVI} = (R_{\text{NIR}} - aR_{\text{red}} - b)/\left(1 + a^2\right)^{1/2}$	Leaf area index	Richardson and Wiegand (1977)
Wide dynamic range vegetation index	$\text{WDRVI} = (0.1R_{\text{NIR}} - R_{\text{red}})/(0.1R_{\text{NIR}} + R_{\text{red}})$	LAI, vegetation cover, biomass	Gitelson (2004)
Soil-adjusted vegetative index	$\text{SAVI} = (R_{\text{NIR}} - R_{\text{red}})(1 + L)/(R_{\text{NIR}} + R_{\text{red}} + L)$	Leaf area index	Huete (1988)
Transformed soil-adjusted vegetative index	$\text{TSAVI} = (aR_{\text{NIR}} - aR_{\text{red}} - b)/(R_{\text{red}} + aR_{\text{NIR}} - ab)$	Leaf area index, biomass	Baret et al. (1989)
Enhanced vegetation index	$\text{EVI} = 2.5(R_{\text{NIR}} - R_{\text{red}})/(R_{\text{NIR}} + 6R_{\text{red}} - 7.5R_{\text{blue}} + 1)$	Leaf area index	Huete et al. (2002)
Green NDVI	$\left(R_{\text{NIR}} - R_{\text{green}}\right)/\left(R_{\text{NIR}} + R_{\text{green}}\right)$	Intercepted PAR, vegetation cover	Buschmann and Nagel (1993), Gitelson and Merzlyak (1994)
Red-edge NDVI	$(R_{\text{NIR}} - R_{\text{red edge}})/\left(R_{\text{NIR}} + R_{\text{red edge}}\right)$	Intercepted PAR, vegetation cover	Gitelson et al. (1996a, b), Gitelson and Merzlyak (1994),
Plant senescence reflectance index (PSRI)	$\text{PSRI} = (r_{0.66} - r_{0.51})/r_{0.76}$	Plant canopy senescence	Merzlyak et al. (1999)
Visible atmospherically resistant indices	$\text{VARI}_{\text{green}} = \left(R_{\text{green}} - R_{\text{red}}\right)/\left(R_{\text{green}} + R_{\text{red}}\right)$	Green vegetation fraction	Gitelson et al. (2002)
	$\text{VARI}_{\text{red edge}} = \left(R_{\text{red edge}} - R_{\text{red}}\right)/\left(R_{\text{red edge}} + R_{\text{red}}\right)$	Green vegetation fraction	Gitelson et al. (2002)
Chlorophyll indices	$\text{CI}_{\text{green}} = \left(R_{\text{NIR}}/R_{\text{green}}\right) - 1$	LAI, GPP, chlorophyll	Gitelson et al. (2003, 2005)
	$\text{CI}_{\text{red edge}} = \left(R_{\text{NIR}}/R_{\text{red edge}}\right) - 1$	LAI, GPP, chlorophyll	Gitelson et al. (2003, 2005)
Modified chlorophyll absorption ratio index	$\text{MCARI} = ((R_{710} - R_{660}) - (0.2 * (R_{710} - R_{560}) * (R_{710}/R_{660})$		Haboudane et al. (2004)
Normalized pigment chlorophyll ratio index (NCPI)	$\text{NCPI} = (R_{660} - R_{460})/(R_{660} + R_{460})$		Fillela et al. (1995)
Triangular greenness index (TGI)	$\text{TG} = -0.5[(\lambda r - \lambda b)(R_{660} - R_{560}) - (\lambda r - \lambda g)(R_{660} - R_{485})]$		Hunt et al. (2011)

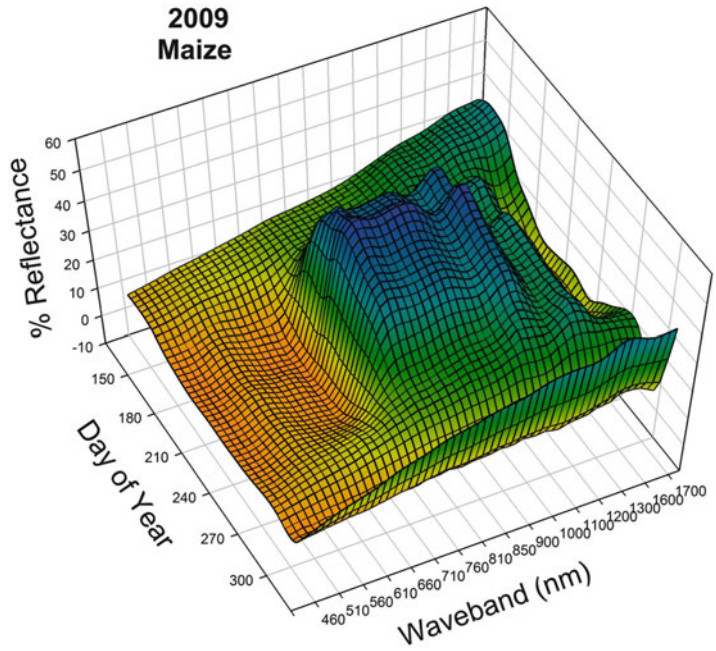

Fig. 14.1 Reflectance changes in the reflectance of a maize canopy throughout the growing season in the wavelengths from 0.5 to 1.7 μm

Changes in reflectance of leaves have been related to changes in nutrient status of the canopy (Filella et al. 1995; Peñuelas et al. 1994). Over the past decade, there has been the continual refinement in the use of leaf chlorophyll indices to estimate nutrient status in different plants with particular attention to maize and wheat (*Triticum aestivum* L.). Chlorophyll change in plant leaves has been one of the indicators of nutrient status, and one example of crop sensing has been applied to nitrogen management using the Soil-Plant Analysis Development (SPAD) chlorophyll meter, color photography, or canopy reflectance factors to assess nitrogen variations across grower's corn fields (Schepers et al. 1992, 1996; Blackmer et al. 1993, 1994, 1996a, b; Blackmer and Schepers 1996). These techniques were based on comparisons with readings obtained from an adequately fertilized strip in the same field in order to eliminate a requirement for establishing the relationship between nutrient concentration and crop reflectance.

In crop sensing, we are often observing the change in leaf or canopy chlorophyll concentrations, and it is important to understand how leaf chlorophyll interacts with light in affecting the reflectance patterns of crops. Remote sensing tools have been constructed on the principle that pigment content strongly affects leaf absorption spectra and is responsible for the patterns observed in reflectance (Fig. 14.1). As the leaf chlorophyll (Chl) content increases, visible wavelength absorption increases, reaching more than 90 % in the blue (0.4–0.5 μm) region by both chlorophyll-*a* and chlorophyll-*b* and carotenoids and the red (~0.67 μm) region where both chlorophylls absorb. Specific absorption coefficients of leaf pigments are high for blue and red wavelengths meaning there is low reflectance in these wavebands (Lichtenthaler 1987) and the depth of light penetration into the leaf is very low. As a result, even low amounts of pigments are sufficient to saturate absorption and cause the reflectance in these wavebands to be constant even with changes in the total amount of leaf area (Fig. 14.1). Even in yellowish-green leaves when Chl exceeds 100 mg/m^2, total absorption exceeds 90 %, and further increases of leaf pigment content do not increase total absorption. This creates a situation in which absorption versus total Chl reaches a plateau, and absorption becomes virtually insensitive to further Chl increases (Thomas and Gausman 1977; Chapelle et al. 1992; Buschmann and Nagel 1993; Gitelson and Merzlyak 1994; Gamon and Surfus

1999). The closer the wavelength is to the main absorption wavelength of pigments (blue or red), the lower the Chl content at which saturation of absorption vs. Chl relationship appears. In the green (0.55 μm) and red-edge (0.7 μm) regions, the absorption coefficient of chlorophylls is very low and seldom exceeds 6 % compared to the blue and red wavelengths (Lichtenthaler 1987). This causes the sensitivity of absorption to Chl content to be much higher in these spectral regions than for the blue and red. Reflectance in the green and the red-edge waveband ranges is sensitive to the whole range of Chl variation with the largest variation in reflectance between 0.53 and 0.59 μm and in the red edge around 0.71 μm. Reflectance varies slightly in the NIR mainly due to leaf internal structure and thickness changes. The observations by Chappelle et al. (1992) and Gitelson and Merzlyak (1994) summarize the relationship between leaf absorption and reflectance as follows: (a) minimum sensitivity to pigment content is in the blue between 0.4 and 0.5 μm and in the NIR; (b) both absorption and reflectance of leaves with moderate-to-high Chl are essentially insensitive to Chl content in the red absorption band of chlorophyll-*a* near 0.67 μm; (c) the green and red-edge reflectance values are closely related for a wide range of leaf greenness; and (d) the highest sensitivity of reflectance and absorption to pigment variation is in the green from 0.53 to 0.59 μm and in the red edge around 0.7 μm. Fundamentally, these relationships have to be understood in order to begin to develop more rigorous methods for the estimation of nutrient status in crop canopies.

14.3 Crop Sensing of Nutrient Status

One of the first comparisons of normal compared to nutrient-deficient maize leaves was reported by Al-Abbas et al. (1974), and they reported that the leaf chlorophyll concentrations decreased with any nutrient deficiency. They observed that nitrogen, sulfur, magnesium, and calcium deficiencies increased the near-infrared reflectance compared to normal leaves in intact maize leaves (Al-Abbas et al. 1974). This fundamental work along with the other research on spectral reflectance has provided the basis for the further development and refinement of crop sensing in precision nutrient management (Moran et al. 1997). The discovery that these changes in spectral reflectance could be used to detect and correct nitrogen deficiency in wheat by Raun and his coworkers at Oklahoma State University has launched a continuum of research since the mid-1990s (Stone et al. 1996; Raun et al. 2002). Their work extended the original observations by Schepers and his coworkers on corn in Nebraska by moving away from the SPAD meter for the assessment of nutrient status into a more robust reflectance index, and this group proposed the use of the normalized difference vegetative index (NDVI) which is expressed as $\mathrm{NDVI} = (R_{\mathrm{NIR}} - R_{\mathrm{red}})/(R_{\mathrm{NIR}} + R_{\mathrm{red}})$. There have been other studies which have proposed the use of a green NDVI (GNDVI) expressed as $\left(R_{\mathrm{NIR}} - R_{\mathrm{green}}\right)/\left(R_{\mathrm{NIR}} + R_{\mathrm{green}}\right)$ (Sripada et al. 2005). This was a departure from earlier use of the SPAD observations or reflectance values related to the chlorophyll content of leaves. It is important to understand the results obtained by the use of the SPAD meter for nitrogen management. Blackmer et al. (1994) compared reflectance in the 0.4–0.7 μm range to the SPAD meter (transmittance at 0.65 and 0.94 μm) and leaf nitrogen concentrations and specific nitrogen content in leaves of maize and found that reflectance at the 0.55 μm wavelength exhibited the most consistent relationship to detect nitrogen differences. They followed this work with an analysis of aerial photography using reflectance of 0.536 μm and found this technique was able to quantify the variability in crop nitrogen status across maize fields. This research team demonstrated that the chlorophyll content corresponding to luxury consumption of nitrogen is dependent on development stage (Blackmer and Schepers 1995). They found that under conditions of luxury consumption of nitrogen, leaf chlorophyll content reaches an upper limit; however, this upper limit changes during the growing season with leaf tissue age. The implications of this observation showed that when nitrogen supply to maize is adequate, reflectance of corn canopies changes little with any

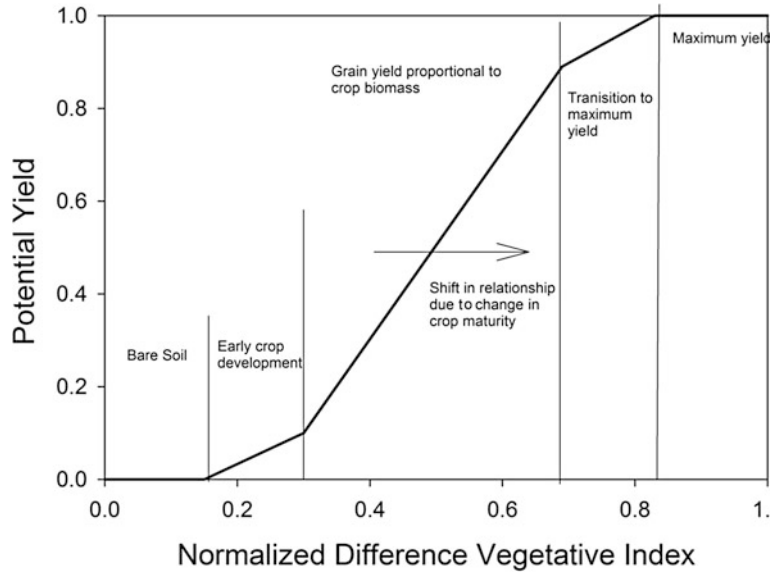

Fig. 14.2 Idealized relationship between the seasonal course of the normalized difference vegetative index and the application to evaluating yield potential in maize and wheat (Adapted from Solie et al. (2012))

increase in nitrogen supply (Blackmer et al. 1996b). These observations on changes in leaf chlorophyll content and its variation also form the basis on why the most reliable use of reflectance observations is from the comparison of a nitrogen-rich strip to other locations in the field (Raun et al. 2001, 2002; Flowers et al. 2003; Hawkins et al. 2007; Shanahan et al. 2008; Kitchen et al. 2010; Roberts et al. 2010; Solari et al. 2010; Scharf et al. 2011; Clay et al. 2012). Scharf and Lory (2002, 2009) used both color film and reflectance measurements to predict economic optimum nitrogen rates (EONR) for maize at the six-leaf stage and concluded that for this method to work, there could be no nitrogen applied at planting, a high nitrogen strip is necessary for comparisons, and there can be no soil background present in any of the observations.

In the past 12 years, there has been an explosion in the amount of research centered on the use of remote sensing methods for estimating nitrogen requirements in corn and wheat with extension to rice (*Oryza sativa* L.) and sugarcane (*Saccharum officinarum* L.). Raun et al. (2011) observed that the difference in the NDVI relative to the growing degree days (GDD) accumulated between the two observation periods was related to the estimated yield in wheat and there was a strong relationship between final grain yield and estimated yield. They found this relationship sound unless there was an event which reduced yield, e.g., delayed harvest to late summer rains or lodging or shattering. Water stress is a major factor affecting nitrogen response in crops, and Clay et al. (2006, 2012) found that water availability affected the utility of a reflectance index and suggested that the use of indices should be constrained to management zones with similar crop water status within a field. In the nitrogen response of cereals, there has been research which has related a change in plant growth to yield, and these changes in growth can be sensed as part of the detection process. One example of this relationship between NDVI and maximum yield in wheat is shown in Fig. 14.2 as proposed by Solie et al. (2012), and they expanded this relationship into a more generalized relationship for the use of the NDVI approach for nitrogen management for both maize and wheat. This relationship provides the rationale for nitrogen management in crops using a reflectance index based on the changes in the biomass. The algorithm developed by Solie et al. (2012) is based on the comparison of the measured reflectance of an area of the field compared to a well-fertilized area. This follows the findings of Blackmer

et al. (1996a) in which the estimation of the nitrogen deficiency was greatly improved when the field area was compared to a nitrogen-rich strip in the field. Their development of the index which utilizes a plateau for a nitrogen-rich crop is very similar to the results obtained by Blackmer and Schepers (1995). The comparison of the observations of leaf chlorophyll or reflectance in the field compared to a nitrogen-rich strip has become a standard operating procedure for utilizing different indices for nitrogen management. This approach has become known as a sufficiency index or relative index. Solie et al. (2012) developed their algorithm for potential yield with nitrogen management, YP_N, based on the following equation:

$$YP_N = \frac{YP_{max}}{1 + \exp[(RI_{NDVI}FP_{NDVI} - Inf)/K]} \quad (14.1)$$

where YP_{max} is the maximum potential yield, Inf is the location of the inflection point of the model where the predicted yield is one-half of the potential yield, and K is the curvature of the sigmoidal model. In their approach they defined FP_{NDVI} as the average NDVI value from an adjacent strip with the nitrogen fertilizer applied at the field or the farmer rate, and RI_{NDVI} is calculated as the NR_{NDVI}/FP_{NDVI} where NR_{NDVI} is the NDVI value from a nitrogen-rich strip in the field. This approach has been used on both wheat and maize with acceptable results. Solie et al. (2012) stated that their generalized model using the NDVI approach with observations collected midseason could be applied to nitrogen fertilizer management in maize and wheat.

The relationship of crop parameters to crop yield has been the focus of several studies associated with improved nutrient management (e.g., Blackmer et al. 1996b; Raun et al. 2001; Freeman et al. 2007; Adami et al. 2010). Yin et al. (2011) demonstrated that plant height in maize was related to grain yield, while Freeman et al. (2007) found there was a strong relationship between maize plant height and biomass and used an index based on NDVI x plant height collected between the eight- and ten-leaf stage. In one experiment, Yin et al. (2011) evaluated six different nitrogen rates—0, 62, 123, 185, 247, and 308 kg·N·ha^{-1}—in four different maize production systems: nonirrigated maize after maize, nonirrigated maize after soybean (*Glycine max* (L.) Merr.), irrigated maize after soybean, and nonirrigated maize after cotton (*Gossypium hirsutum* (L.)]. Maize yield was significantly related to plant height and positive at all three vegetative growth stages, i.e., 6-leaf (V6), 10-leaf (V10), and 12-leaf (V12). There was an exponential relationship between plant height and grain yield with the strongest relationship at the V10 and V12 growth stages which was consistent among the management systems (Yin et al. 2011). There was an effect of nitrogen rate on the plant height at each of the growth stages, and these authors suggested that the use of ultrasonic plant height detectors could potentially be used to improve nitrogen management in maize. In a subsequent study, Yin and McClure (2013) found the relationships among maize yield, plant biomass, plant height, and leaf nitrogen combined with NDVI for the same four management systems used by Yin et al. (2011) varied among years. They suggested that the use of NDVI with plant height is variable when used in the early to mid-season growth periods, and there were instances when both NDVI and plant height provided the best relationships to maize growth, plant nitrogen requirements, and grain yield.

In wheat, tiller density responds to changes in nitrogen availability, and Flowers et al. (2001) related tiller density to spectral indices at the early stage of tiller development and found tiller density was significantly related to near-infrared digital counts. In order to develop a consistent relationship among the locations, relative near-infrared counts were compared to relative tiller density using an approach in which areas with high tiller density were used as a normalizing process (Flowers et al. 2001). This procedure was verified in a subsequent experiment by Flowers et al. (2003) using different wheat varieties, soil colors, and weed densities, and they found that they could provide an accurate nitrogen recommendation 85.5 % of the time across a wide range of environments and management systems. Utilization of the concept of

evaluating tiller density in wheat is similar to the biomass approach by Solie et al. (2012) because changing tiller numbers would account for changing biomass amounts. Mullen et al. (2003) developed a relationship between a response index, $RI_{harvest}$, and RI_{NDVI} in which the $RI_{harvest}$ values measured the responsiveness to additional nitrogen in the grain yield and RI_{NDVI} provided a measure of the response during the growing season. They found $RI_{harvest}$ was accurately predicted ($r^2 > 0.56$) by RI_{NDVI} values when relative values between a non-limiting nitrogen strip and the actual strip were compared. For effective nitrogen management, it is critical to have a comparison strip which represents a non-limiting nitrogen environment within the same field and to avoid any water stress conditions as suggested by Clay et al. (2012). They expanded this comparison of well-fertilized and well-watered wheat strips with the underfertilized area to create a sufficiency index expressed as ratio of NDVI values from a well-fertilized and well-watered area ($SI\text{-}NDVI_{wf}$) compared to a ratio of NDVI from well-fertilized water-stressed areas ($SI\text{-}NDVI_{mz}$). In their experiment they found that water and nitrogen stress at stem extension and flag leaf stage increased green, red, and red-edge reflectance and reduced the NDVI values. The use of the $SI\text{-}NDVI_{mz}$ values had a greater accuracy in fertilizer recommendations than the $SI\text{-}NDVI_{wf}$ values (Clay et al. 2012). Sripada et al. (2007) used a similar approach at the end of the tillering stage in wheat using color infrared photography and found that when the biomass was greater than 1,000 $kg{\cdot}ha^{-1}$, the best indicator was based on the comparison of measured reflectance compared to a high nitrogen reference strip.

The findings by Mullen et al. (2003) suggest that remote sensing techniques can provide a technique for identifying environments where responsiveness to nitrogen has provided a foundation for subsequent research to improve nitrogen management. Sripada et al. (2008) and Barker and Sawyer (2010) utilized active sensors to compute the nitrogen needed to sustain maize yields using a number of different indices. Barker and Sawyer (2010) used a comparison as a differential from the economic optimum nitrogen rate (dEONR) with different sensor indices, computed NDVI based on green wavebands, NDVI, SPAD, and other waveband combinations from these active sensors. Barker and Sawyer (2010) found that the variability in prescribed nitrogen rate was largest as the difference from the EONR was small because when nitrogen deficiency is small, the detectable differences in leaf area or biomass from these spectral indices are also small. In a subsequent study, Barker and Sawyer (2010) used the normalized green NDVI index to evaluate nitrogen management strategies in maize in which there was a range of preplant nitrogen applications from 0 to 270 $kg{\cdot}N{\cdot}ha^{-1}$ and observations were made at the ten-leaf stage of development. They used the GNDVI values to determine the nitrogen rate to apply and found that preplant nitrogen plus the sensor-applied nitrogen created the maximum maize yield recovery from nitrogen stress. In this study, yield recovery from deficit nitrogen occurred when there was limited preplant nitrogen applied and the GNDI values were used to estimate nitrogen application rates (Barker and Sawyer 2010). They concluded that the method of preplant nitrogen with the GNDVI method for determining additional nitrogen to apply would provide maize producers with a management tool to avoid nitrogen deficiency and obtain potential yields (Barker and Sawyer 2010). Sripada et al. (2005) used a similar approach in which they applied different amounts of nitrogen to maize at planting and at tasseling and then collected yield and near-infrared color photography just before the nitrogen application at tasseling. They found they could predict EONR at the tasseling stage using a relative GNDVI value to a high nitrogen strip and could utilize this method to improve nitrogen management. Sripada et al. (2006) evaluated relative GNDVI at the seventh leaf stage and found significant relationships with nitrogen application rates; however, the scatter about the linear relationships was large. Sripada et al. (2008) demonstrated the method of using the relative differences in reflectance with the GNDVI and coupled this with the ratio of nitrogen fertilizer cost to corn price to adjust the EONR for maize. There are advantages to using these techniques to

improve nitrogen management during the growing season for use by producers.

The current methodology to estimate nitrogen response has been the direct comparison between a nitrogen-rich strip and the other areas of the field. Raun et al. (2008) proposed the use of a ramp calibration method for preplant nitrogen application rates as a method to determine the crop responsiveness over a range of application rates using 16 different incremental nitrogen rates from 0 to 225 kg·N·ha^{-1} over a 55 m strip in the field. Observations of NDVI would be collected over different treatments to determine the nitrogen rate where the NDVI values are maximum across the ramp calibration strip. Using this approach allowed for the determination of nitrogen response at the Feekes growth stage 5 in wheat and the eight-leaf growth stage in maize. This system may provide a more reliable indication of nitrogen response than the nitrogen-rich strip because of the lack of responsiveness observed when the rates are near the EONR (Barker and Sawyer 2010). Scharf et al. (2011) compared the sensor-based nitrogen application rates relative to the producer's selected nitrogen rates across 55 replicated on-farm trials in Missouri and found the use of the sensor-based rates based on a relative visible/near-infrared index compared to a nitrogen-rich strip increased the profit by $42 ha^{-1}, increased yield by 110 kg·ha^{-1}, and reduced the nitrogen application by 16 kg·ha^{-1}. They concluded that the use of the sensor-based methods for nitrogen management was superior to the producer's selected nitrogen rates (Scharf et al. 2011). The use of reflectance indices of different forms using either active or passive sensors has proven to be a reliable method for determining nitrogen application rates to maximize yields provided that the observations obtained within a field are compared to a nitrogen-rich strip within the same field. The recent results by Arnall et al. (2013) obtained from 261 site years of long-term wheat and maize studies across the Midwestern United States the nitrogen responsiveness or the response index by dividing the actual grain yield from high nitrogen rate plots with either the 0 nitrogen fertilizer check or the medium nitrogen rate plots. They found that nitrogen responsiveness and yield potential were independent of each other and a necessary step for the development of more accurate sensing of determination of fertilizer rates to be adjusted at mid-season will need both potential yield and the response index as independent variables.

The relationships developed between different indices based on reflectance observations show promise in being able to detect nitrogen deficiency with sufficient accuracy to allow for improved nitrogen management. The relationships reported by the different studies show a wide range in the strength of the relationships; however, the continual refinement over the course of the past decade has made these techniques a viable management tool for wheat and maize production.

14.4 Application to Other Crops

The vast majority of the research and application of remote sensing to nitrogen management has been conducted and evaluated on maize and wheat production systems; however, there have been many studies conducted on other crops as well. Bronson et al. (2005) evaluated the NDVI approach for cotton in the Texas High Plains and found that NDVIs were poorly related to leaf nitrogen content, plant biomass, and lint yield. They concluded that for this approach to work, there must be a use of the well-fertilized areas and the sufficiency index. Zhao et al. (2005) found that leaf chlorophyll was closely related to reflectance ratios using either R_{708}/R_{915} or R_{551}/R_{915} wavebands and there was a linear relationship between leaf nitrogen content and the reflectance ratio of R_{517}/R_{413}. In a subsequent study based on the observations from their original study, Bronson et al. (2011) found that when NDVI values in the field decreased below a nitrogen-rich area and nitrogen was added to the treatment areas, there was a reduction in the amount of nitrogen applied by 33 % with no impact on lint or seed yield. They also found that the EONR varied from 23 to 75 kg·N·ha^{-1} among the 3 years of their study suggesting that the nitrogen response will differ among years due to growing conditions. There are differences in

the results obtained from these studies on cotton; however, the overall conclusion is that reflectance-based approaches provide a viable method for nitrogen management in cotton (Raper et al. 2013).

In rice (*Oryza sativa* L.), Turbaña et al. (2011) found that NDVI and a simple ratio of R_{NIR}/R_{red} varied in their relationship to biomass and grain yield depending upon the viewing angle of the sensor. At panicle differentiation, there was no difference in the relationship across viewing angles; however, at 50 % heading the off-nadir angles viewed more green vegetation. Both the NDVI and simple ratio method produced a large variation in the goodness of fit among years indicating that without a reference method for nitrogen, single measurements will not produce reasonable results. When they utilized a nitrogen-rich strip as part of the observational procedure and then compared a relative NDVI with a relative yield, then there was a significant linear relationship between relative NDVI and relative yield values ($r^2 = 0.63$) for the observations collected at panicle differentiation plus 1 week (Turbaña et al. 2012). Lee et al. (2008) had previously observed that the ratios of different wavebands and the NDVI did not provide as reliable indicator of leaf N concentration or accumulations as compared to a derived spectral model based on the reflectance at 0.735 μm. They concluded from their studies that the use of this approach coincides with the current schedule for sidedress applications of nitrogen in the southern United States and could provide a valuable management tool.

Lofton et al. (2012) applied the same approach of using a relative NDVI and a relative yield response for sugarcane and found a strong relationship between the response index and cane tonnage ($r^2 = 0.92$) and sugar yield ($r^2 = 0.81$). They concluded that this approach is a method for evaluating sugarcane response to nitrogen fertilizer management and the adaptation of methods developed for other crops are applicable to sugarcane (Lofton et al. 2012). Gehl and Boring (2011) found a similar response in sugar beets (*Beta vulgaris* L.) when the NDVI approach was used and could be used as a tool to estimate yield and recoverable sugar. Flowers et al. (2010) evaluated the NDVI approach for seed production in perennial ryegrass (*Lolium perenne* L.), and relative values of NDVI or near-infrared were comparable to the relationship between seed production and tissue testing of leaf nitrogen concentration. The application of the methods of using relative NDVI or relative GNDVI values between a nitrogen-rich strip and the treatment area performs equally well for crops other than maize and wheat; however, each crop requires the development of the relationship between crop yield and biomass to determine the nitrogen management response relationships.

14.5 Development of New Methods of Crop Sensing

Bélanger et al. (2005) proposed the use of a multivariate indicator to detect nutrient deficiencies in crops and proposed for potato (*Solanum tuberosum* L.) that a multivariate indicator of nitrogen imbalance (MINI) based on reflectance and fluorescence indices would be useful. They used reflectance indices based on combinations of wavebands and simple ratios and found that MINI could correctly detect 70 % of the nitrogen-deficient plants and 90 % of the nitrogen-sufficient plants, and this method would be more rapid than the current method of tissue testing. This approach has been utilized in a nitrogen management system but demonstrates the utility of remote sensing indices to quantify nitrogen deficiency in crops. However, this type of approach may require extensive calibration to assess its utility at the canopy scale. The original research on nutrient deficiency focused on the chlorophyll content in the leaf, and the utilization of the NDVI or GNDVI has replaced leaf chlorophyll to focus more on remote sensing parameters more closely related to canopy biophysical properties, e.g., canopy biomass or potential crop yield. One example of utilizing additional portions of the light spectrum was shown by Adami et al. (2010) in which they linked the NDVI values with absorption

parameters of a wheat canopy at 1.025 μm to obtain improved estimates of leaf area index and grain yield.

There have been several chlorophyll indices developed to directly estimate the chlorophyll content of plant canopies. These range from the simple ratio index proposed by Gitelson et al. (2003, 2005) as $CI_{green} = \left(R_{NIR}/R_{green}\right) - 1$in which the value of 1 in the relationship is the soil background offset. This value will vary among soils, and for Ames, Iowa, on a Nicollet soil with 4 % organic matter in the surface, the value is 1.6. There are other chlorophyll indices which have been developed including the modified chlorophyll absorption ratio index (MCARI) developed by Fillela et al. (1995), the normalized pigment chlorophyll ratio index (NPCI) developed by Haboudane et al. (2004), and the triangular greenness index (TGI) by Hunt et al. (2011) with their equations shown in Table 14.1. There may be more value in exploring the use of alternative remote sensing methods for estimating nitrogen response compared to the NDVI method. A comparison of these four methods for a nitrogen study on corn is shown in Fig. 14.3 as an example of the seasonal variation in two different nitrogen management practices of no nitrogen fertilizer and 150 $kg{\cdot}ha^{-1}$ of preplant nitrogen. During the period in which the NDVI method is used, the NDVI method is one of the least responsive to differences in nitrogen supply compared to any of the other three indices (Fig. 14.3). Eitel et al. (2008) focused on chlorophyll-*a* and chlorophyll-*b* and nitrogen status in wheat in Idaho and Oregon using a number of simple indices which are currently being used for nitrogen management assessment and found that NDVI, GNDVI, and simple ratio using green, red, or near-infrared-reflectance were poorly related to leaf nitrogen concentration or to SAPD readings because these indices were extremely sensitive to changes in leaf area index. This confounded the ability to detect nitrogen status in the crop and found the MCARI/ MTV12, as proposed by Eitel et al. (2007) where MTV12 is the modified triangular vegetation index $(MTV12 = 0.5[120(R_{700} - R_{550}) - 200(R_{670} - R_{550})$, to provide the most consistent relationship to either SPAD or leaf nitrogen tissue concentration.

An alternative index for chlorophyll was proposed by Cammarano et al. (2011) in which they used the normalized difference red edge (NDRE) computed as $(R_{790}–R_{720})/(R_{790}–R_{720})$ and the crop canopy chlorophyll index (CCCI) expressed as the $(NDRE–NDRE_{min})/(NDRE_{max}–NDRE_{min})$. Their approach is similar in function to previous work in using a nitrogen-rich strip as the basis of the comparison but still utilizes remote sensing information of the canopy. In their studies the goodness of fit for the nitrogen management model was good with a RMSE of 0.2 $g{\cdot}N{\cdot}m^{-2}$ in the model (Cammarano et al. 2011). Delegido et al. (2011) used a detailed reflectance data to derive a normalized area over reflectance curve (NAOC) expressed as follows:

$$NAOC = 1 - \frac{\int \frac{795}{643} R d\lambda}{152 R_{795}} \tag{14.2}$$

where R is the reflectance between the two wavelengths and 152 represents the maximum reflectance at R_{795}. They used this model to obtain chlorophyll observations closely related to SPAD readings across a number of crops growing in the Iberian region of Spain (Delegido et al. 2011). Adams et al. (2000) used a yellowness index (YI) based on a graphical method to determine the concavity or convexity of concentrations of leaf chlorophyll induced by different nutrients which quantifies leaf chlorosis. The approach is based on the wavelength range between 0.55 and 0.67 μm and is the second derivative of the reflectance spectrum between these two wavelengths. This has only been evaluated on single leaves in controlled systems but does offer the potential to provide a different method to quantify leaf chlorosis.

There continues to be the development of techniques which provide different methods of utilizing remote sensing for the detection of crop nitrogen status. The current methods rely heavily

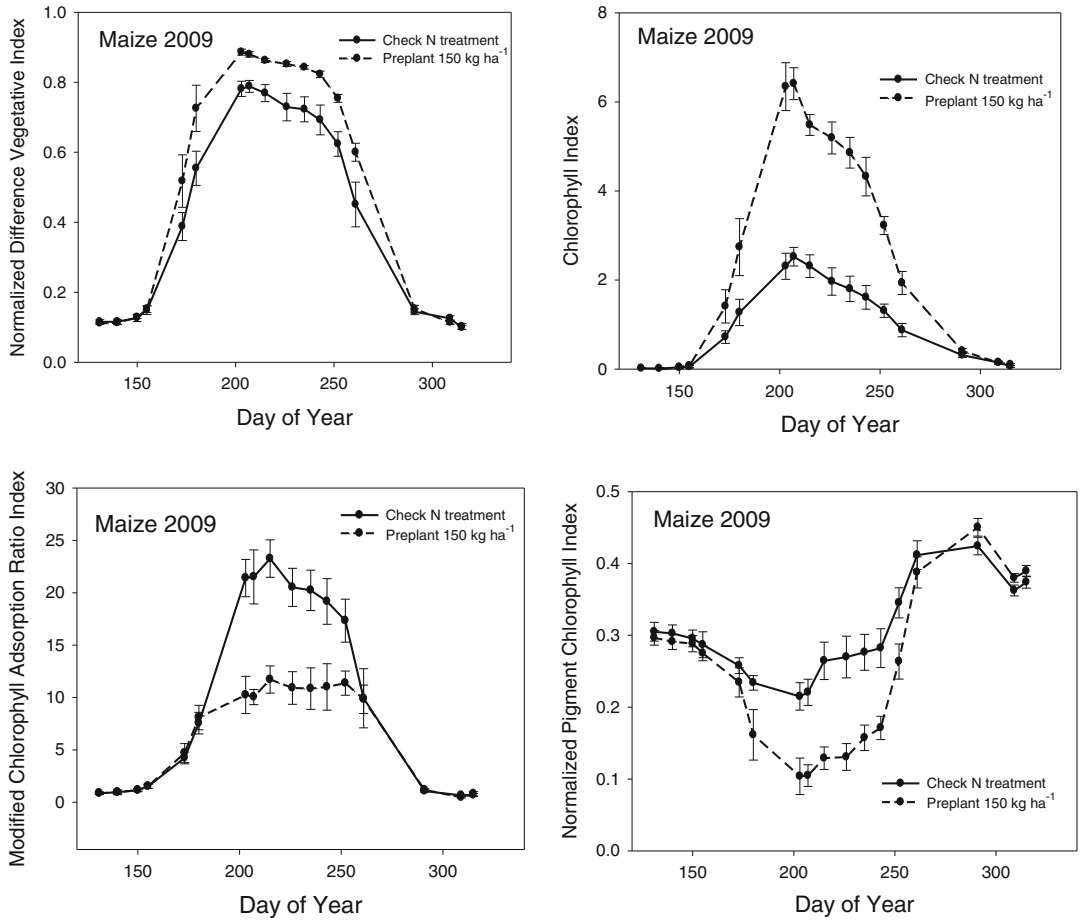

Fig. 14.3 Comparison of normalized difference vegetative index (*NDVI*), chlorophyll index, modified chlorophyll adsorption ratio index, and normalized pigment chlorophyll index over the growing season for maize grown in Ames, Iowa, under two different nitrogen management regimes. The period from day of year 169–183 represents the V6–V10 period of phenological development

upon a nitrogen-rich strip, and as found by Barker and Sawyer (2010), the ability to resolve differences may be limited when only minimal amounts of nitrogen are needed. The findings of Clay et al. (2012) suggest that comparisons to a nitrogen-rich strip may require isolating these comparisons to the same management zone within the field because of the differences in soil water availability creating problems in accurately detecting nitrogen stress. However, Inman et al. (2008) demonstrated that the inclusion of soil management zones based on color along with NDVI across fields resulted in only marginal improvement in their ability to assess maize yields. Addressing the problems of heterogeneous fields will offer a challenge to being able to effectively develop nitrogen management strategies (Zillmann et al. 2006). The development of methods to continue to refine and improve nutrient management would be of agronomic benefit.

14.6 Application of Crop Sensing and the Impact

The ultimate goal of the crop sensing techniques is to improve agronomic management in different crops. There have been several metrics which include increased yield, decreased nitrogen

input, or increased nitrogen use efficiency. Hong et al. (2006) demonstrated agronomic and environmental benefits with the utilization of the remote sensing methods to determine nitrogen needs of the crop. They found there was a reduction in nitrate-N (NO_3-N) while reducing nitrogen inputs and increasing the harvest nitrogen ratio in both maize and wheat. This system was more efficient than the best management practice recommended for this region of North Carolina. These observations support the conclusions of Ferguson et al. (2002) that utilization of remote sensing methods for site-specific management of maize would reduce residual NO_3-N and would be a potential method to reduce groundwater contamination from NO_3-N. Kitchen et al. (2010) found the value of using the canopy reflectance method to obtain NDVI values and a sufficiency index increased as the price of nitrogen fertilizer increased and the profits increased between \$25 and \$50 ha^{-1}.

In a different approach to using remote sensing information, Kyveryga et al. (2010) used aerial imagery to guide the late season fall nitrate test in maize as a means of evaluating the effectiveness of different nitrogen management practices. This methodology offers a method for quantifying the value of adaptive management strategies in agricultural systems. Overall, the utilization of crop sensing provides a framework for the improvement of nitrogen management both spatially and temporally across fields.

14.7 Challenges

There have been great advances in the past 10 years in the development, evaluation, and refinement of the approaches used to detect nutrient deficiency in the field with crop sensing techniques. These efforts have focused primarily on nitrogen because of the variable response and the potential for environment impacts from excess nitrogen; however, with continued research the detection of deficiencies of other nutrients is feasible. Although, there have been advances in the evaluation of both the SPAD and NDVI or GNDVI approaches, the challenge remains to demonstrate that these techniques can be incorporated into precision agricultural management systems. The current research results show success at the small field scale with advantages in improving profit, reducing nitrogen application amounts, improving nutrient use efficiency, and enhancing environmental quality. Nevertheless, these methods have yet to be extended to an operational scale where the effectiveness could be determined over large land areas.

The current methods use either SPAD meters, simple ratios of wavebands, or the NDVI or GNDVI methods as the foundation and require the use of a reference strip within a field for the direct comparison between a nitrogen-rich area and the area to be managed. The SPAD system is based on leaf chlorophyll; however, many of the other spectral methods are more closely related to leaf biomass or leaf area and build their relationships to nutrient management on a comparison of changes in these canopy properties rather than leaf or canopy nitrogen concentration. The challenge should be to continue to develop spectral models for potential use in precision agriculture which are more closely linked to changes in chlorophyll than biomass. This will create systems which may rely less on a comparison strip in the field and more on actual plant response to nutrient deficiencies.

Agriculture faces many challenges to increase the efficiency of production, and further development of techniques to improve management will pay large dividends from both a production and an environmental endpoint. Addressing these challenges will provide a more robust agricultural system, and the progress made in the past 10 years offers the promise for continued advances in the next decade.

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Phenotyping Nutritional and Antinutritional Traits

15

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Abstract

Evolution of nutrient-rich food systems to calorie-focused production agriculture has created serious agricultural and human health issues: marginalization of traditional agricultural crops, greater dependence of agricultural inputs, and creation of both energy and micronutrient malnutrition. To date more than half of global human populations are suffering numerous health problems associated with excess calories and lack of essential micronutrients. Pulse crops, in particular lentils, are promising crops not only to improve human health but also to reduce agricultural inputs toward greater agricultural sustainability. In this book chapter, human micronutrient malnutrition issues, suggestions to reduce micronutrient deficiencies, promise of pulse crops using lentil as an example, lentil's micronutrient and antinutrient profiles, nutrient analytical procedures, and the needs to shift our thinking from calorie-focused to nutrient-focused approaches are also presented.

Keywords

Micronutrients • Malnutrition • Biofortification • Fe • Zn • Foliates • Methods of analysis

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15.1 Introduction

Millions of people around the world suffer from appalling nutrition. To combat global malnutrition, novel ways to produce nutritious foods, beyond calorie-focused approaches, are required. Intensive agricultural practices resulting from the "green revolution" increased production of cereal crops, mainly rice (*Oryza sativa* L.), wheat (*Triticum* spp.), and maize (*Zea mays* L.), and have enabled most Asian and African

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populations to get enough calories from their staple foods. However, while cereal staples are a good source of carbohydrates and can satisfy daily caloric requirements, they do not provide daily requirements of protein and a range of micronutrients including iron (Fe), zinc (Zn), iodine (I), vitamins A and C, riboflavin, selenium (Se), copper (Cu), calcium (Ca), folates, and carotenoids. Proteins and micronutrients are not only essential for general well-being; they are also being increasingly recognized as important for human health and disease prevention. Continuing to follow intensive cereal- or "calorie"-only food production practices may further increase the number of malnourished people around the world. Malnourished populations have weak physical and mental growth potential, have reduced work productivities, and continue to suffer from other health problems (Welch and Graham 1999, 2004). The inability to find a solution to micronutrient malnutrition or "hidden hunger" could have irreversible impacts on human health and lives into the future.

During the green revolution, calorie-focused cereal crop production practices replaced a range of traditional food crops, including legumes, tubers, fruits, and vegetables; these are the foods that provided micronutrients to many populations of Southeast Asia and Africa (Welch and Graham 2005). The global population continues to increase, with more than 90 million people to feed each year; global food demands are expected to double by 2050. With limited arable lands, decreasing soil fertility, and declining water resources, the present food systems are already challenged with respect to providing sufficient micronutrients to most global populations. Dependence on animal products for daily nutrients is not an option for most populations in the developing world and is becoming more difficult in most developed countries. Therefore, investigating the potential of traditional food crops may be necessary to provide better nutrition solutions toward improved human health.

Food legumes could be a central part of future sustainable food systems. Development of whole grain legumes and other nutritious crops may address malnutrition epidemics in both developing and developed countries. To this end, lentil (*Lens culinaris Medik*), a cool season food legume, has been a central part of biofortification research efforts due to its superior nutritional profile and short cooking time. This chapter focused on nutritional and antinutritional traits and their phenotyping. Nutritional traits include levels of minerals (e.g., Fe), antinutrients (phytic acid and phenolics), prebiotic carbohydrates, and folates. Lentil is discussed as an illustrative example of pulse crop in the context of recent scientific literature.

15.2 Present Status of Micronutrient Malnutrition

Approximately 40 % of the world's population is currently facing hidden hunger due to diets that are deficient in essential micronutrients. Worldwide, 40 % of women and 50 % of pregnant women are Fe deficient; these are staggering statistics when combined with evidence that approximately 40 % of total childbirth deaths could be prevented by adequate Fe status (Welch and Graham 2005). Severe vitamin A deficiency blinds approximately half a million children in Southeast Asia and Africa each year. Zinc and Se deficiencies reduce the body's ability to fight off malaria, diarrhea, pneumonia, and HIV. Rickets, a forgotten skeletal disorder due to vitamin D or calcium deficiency that results in the inadequate mineralization of the bone, has reemerged as a public health problem in Bangladeshi infants and children. To address these nutritional problems through food systems, the world may need a second revolution: a "greener" revolution to provide not just food but more nutritious foods, including grain legumes. The measureable outcomes would be healthy communities instead of crop production per capita.

Micronutrient malnutrition is defined as a deficiency in one or more vitamins or minerals essential for human health, of which more than 50 have been identified. The three most commonly studied micronutrient deficiencies in the

world are anemia (related to dietary Fe), vitamin A, and iodine (I). Other common micronutrient deficiencies, including zinc (Zn), selenium (Se), and vitamin B_{12}, are equally important for human health (Welch and Graham 2005). The global prevalence of iron-deficiency anemia among children is nearly 47.8 %, and for vitamin A and iodine deficiencies, it is 30.7 and 30.3 %, respectively (WHO 2008). Welch and Graham (2005) argued that this alarmingly increasing micronutrient malnutrition trend is due to the decreased production of micronutrient-rich foods, including pulses (lentils, field peas, chickpea, and common beans) (Welch and Graham 2005). For thousands of years, farmers adopted simpler rotations of high-yielding and more profitable cash crops, i.e., wheat, maize, and rice, with nutritionally rich legume crops to sustain their livelihoods and their societies. However, present-day calorie-centric agriculture is devoid of these traditional or more diverse cropping systems, leading to never-before-seen nutrition transitions that are linked with increased rates of noncommunicable diseases in both developing and developed countries.

Micronutrient deficiencies have been addressed through food fortification and mineral supplementation; however, these efforts have not resulted in reduction of micronutrient epidemics in most parts of the world. Expert recommendations suggest improving human nutritional status through the following efforts: (1) biofortification, i.e., breeding micronutrient-enriched staple food crops through conventional plant breeding and modern biotechnology; (2) diet diversification (includes more pulses, fruits, tubers, small fish, and vegetables); (3) reduction of micronutrient losses from post-harvest and food processing through increased food utilization and accessibility; (4) changing of the combination or mix of food choices to increase micronutrient bioavailability (nutrient absorbance promoters and inhibitors); and (5) recycling of food waste and water (Welch et al. 1997; Bouis and Welch 2010; WHO 2008).

15.3 Biofortification

Past attempts to address hidden hunger have included dietary supplements, food fortification, and, more recently, "biofortification" (breeding crops for increased micronutrient content and bioavailability using conventional breeding and modern biotechnology). Unlike supplementation and fortification, which add ongoing costs to consumers, biofortification offers a unique opportunity to change crop nutritional value within the food system and in ways that have minimum impact on consumer cost. For this reason, biofortification is seen as having great potential as a sustainable, food-based solution to global micronutrient malnutrition (HarvestPlus 2013). Biofortification programs, such as HarvestPlus, BioFORT Brazil, and HarvestPlus-China, have been a tremendous success in Africa, South America, and Asia. These programs collaborate with many scientific institutions, government agencies, and nonprofit agencies around the world. Target crops include rice, wheat, maize, common bean, sweet potato, cassava, and pearl millet for phase one; many other crops are included in phase two. The targeted nutritional traits include Fe, Zn, and provitamin A (Table 15.1). The HarvestPlus program impact pathway has discovery, development, and delivery as its three major phases (Fig. 15.1; Saltzman et al. 2013; Bouis et al. 2011). The discovery phase includes identification of target populations, setting required nutrient targets, validating nutrient targets, and then screening the appropriate germplasm for possible micronutrient traits. The next step is the development of micronutrient-enriched varieties, improving and evaluating these varieties, testing nutritional efficacy, and studying social aspects including farmer adaptation and consumer acceptance. Finally, the new varieties are delivered by release in target countries with concurrent dissemination of information to promote consumption (Fig. 15.1).

The biofortification (nutrient density) and bioavailability of micronutrients are equally important for achieving the optimal micronutrient

Table 15.1 Biofortified crops, target nutrition/agronomic traits, and country-release schedule

Target crop	Nutritional trait	Agronomic trait	Countries	Release schedule
Sweet potato	Provitamin A	Disease/drought/acid soil tolerance	Uganda, Mozambique, Brazil, China	2002–2010
Bean	Fe, Zn	Virus/heat/drought tolerance	Rwanda, DR Congo, Brazil	2008–2012
Pearl millet	Fe, Zn	Mildew/drought/disease tolerance	India	2012
Wheat	Zn	Disease/lodging resistance	India, Pakistan, China, Brazil	2011–2016
Maize	Provitamin A	Disease resistance	Zambia, Nigeria, Brazil, China	2012–2015
Rice	Fe, provitamin A	Disease/pest/cold/submergence	Bangladesh, India, China	2010–2013
Cassava	Provitamin A	Disease tolerance	Nigeria, Kenya	2017
Lentil	Fe, Zn	Disease/heat/drought tolerance	Nepal, Bangladesh, Ethiopia, India	2012
Cowpea	Fe, Zn	Disease	India, Brazil	2008
Banana	Provitamin A, Fe	Unknown	Uganda	2019
Pumpkin	Provitamin A	Unknown	Brazil	2015
Irish potato	Fe	Unknown	Rwanda, Ethiopia	Unknown

Adapted from Saltzman et al. (2013), Miller and Welch (2013), Bouis et al. (2011)

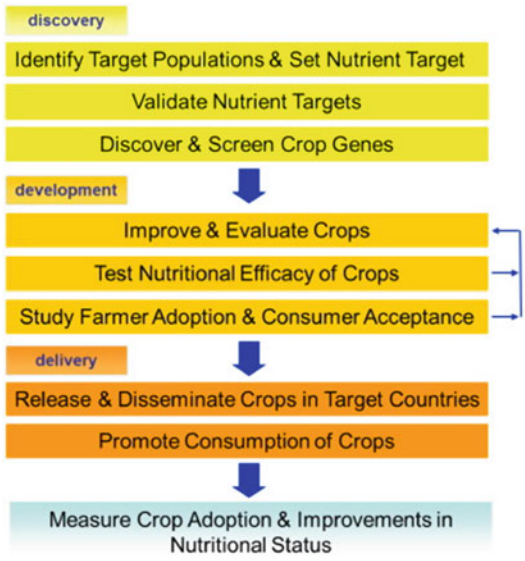

Fig. 15.1 HarvestPlus impact pathway (Adapted with permission from Saltzman et al. (2013))

status of a diet to combat global hidden hunger. Nutrient density is a measure of nutrients in a food per calorie or per unit weight. In contrast, bioavailability is the proportion of an ingested nutrient that is absorbed and utilized for essential metabolic functions. Therefore, bioavailability is a far more important concept with respect to achieving biofortification program goals (Welch 2002; Miller and Welch 2013). The bioavailability of a mineral micronutrient is governed by many factors, including the host, digestive environments, and the presence of mineral absorption promoters and inhibitors in a food. Absorption promoters, such as ascorbic acid, carotenoids, prebiotic carbohydrates, certain fibers, sulfur amino acids, and meat factors, increase Fe absorption in the human digestive system; phytic acid and polyphenols in plant-based food are the major inhibitory factors of Fe and Zn bioavailability (Table 15.2).

15.4 Nutritional Traits for Phenotyping

15.4.1 Fe Bioavailability in Lentils

As presented in Table 15.2, determining the total amount of Fe in seeds is not enough to predict the impact of consumption with respect to meeting human dietary requirements. The bioavailable Fe is the only true measure of the nutritional impact of any Fe-rich food crop. Measurement of human

Table 15.2 Micronutrient bioavailability promoters and inhibitory substances in foods

Nutrient	Promoter substances	Antinutrients (inhibitors)
1. Fe and Zn	1. Organic acids (ascorbic acid, malate, citrate, fumarate)	1. Phytic acid
	2. Amino acids (methionine, cysteine, histidine, lysine)	2. Polyphenols (kaempferol, gallic acid, chlorogenic acid)
	3. Beta-carotene	3. Tannins
	4. Meat factors	4. Certain fibers
	5. Prebiotic carbohydrates (fructooligosaccharides, galactooligosaccharides, sugar alcohols, raffinose family oligosaccharides)	5. Heavy metals
	6. Some phenolic acids-quercetin and ferulic acid	
	7. Hemoglobin (only for Fe)	
	8. Long-chain fatty acids (palmitate; only for Zn)	
2. Se	9. I	
3. Vitamin A	10. Beta-carotene	
	11. Fats and lipids	

Adapted from Welch and Graham (2005), Johnson et al. (2013a)

bioavailability of Fe from plant-based foods is a complex process because numerous factors influence the final Fe bioavailability in the gut: (1) crop genetic selection and production practices; (2) other meal composition; (3) individual characteristics; (4) processing, cooking, and preparation of foods; and (5) individual ingestion, absorption, and utilization of bioavailable Fe. As a result of this complexity, the data obtained from bioavailability models are unclear (Welch and Graham 2005; House 1999; Van Campen and Glahn 1999). Human efficacy trials are the most appropriate way to test for true Fe bioavailability; however, it is impractical to test the bioavailability of Fe of thousands of genotypes that can be generated from breeding programs.

Lentil breeding and nutritional quality programs in the USA and ICARDA together developed a robust model to select appropriate lentil germplasm for Fe biofortification based on food matrix factors. First, Fe bioavailability in lentil germplasm is screened based on food matrix factors, including Fe bioavailability promoters (ascorbic acid, prebiotic carbohydrates, phytoferritin, and carotenoids) and inhibitors (phytic acid, kaempferol, gallic acid, and chlorogenic acid). Once appropriate breeding efforts are carried out, based on the phenotyping data and nutritional quality, Fe bioavailability studies are conducted using an in vitro Caco-2 cell model for selected advanced breeding lines only. Finally, these selected varieties are used in human trials to test the true Fe bioavailability of lentils.

Phenotyping of lentil for true Fe bioavailability is an expensive process, as bioavailability is governed by many food matrix factors. Available data on lentil Fe biofortification are based on the total Fe concentration and the direct measurement of Fe bioavailability using Caco-2 cell culture models (Table 15.3; Thavarajah et al. 2009a; Boum et al. 2008; Della Valle et al. 2013a, b) in the seeds and do not consider food matrix factors or regular household cooking methods that can govern the true Fe bioavailability. Some studies report phytic acid concentration but other food matrix factors are not considered. A recent study reports lentil genotypes with superior Fe concentrations with phenolic and phytic acid profiles for enhanced Fe bioavailability (Table 15.3; Johnson et al. 2013a). Ten lentil commercial cultivars grown in two locations and years were tested for total Fe, phytic acid, ascorbic acid, gallic acid, and chlorogenic acid. Total lentil seed Fe concentration across genotypes was 56–70 mg/kg with low concentrations of phytic acid (6.3–8.7 mg/kg).

Table 15.3 Seed Fe (mg/kg), phytic acid (PA; mg/g), ascorbic acid (AA; mg/kg), gallic acid (GA; mg/kg), and chlorogenic acid (CLA; mg/kg) concentrations as well as Fe bioavailability (using Caco-2 cell culture model; ng ferritin/mg protein) of lentils grown in the USA and Canada

Genotype	Fe	PA	AA	GA	CLA	Fe bioavailability	Reference
CDC Greenland	56.7	8.7	69.4	24.4	20.9	–	Johnson et al. (2013a)
	61.0	6.1	–	–	–	12.2	Della Valle et al. (2013a)
CDC Lemay	67.9	8.5	67.2	24.4	12.1	–	Johnson et al. (2013a)
	75.3	–	–	–	–	3.4	Della Valle et al. (2013a)
CDC Red Rider	56.3	8.4	62.4	22.8	16.8		Johnson et al. (2013a)
	53.4	–	–	–	–	2.5	Della Valle et al. (2013b)
CDC Redberry	56.8	7.3	71.5	22.2	14.8	–	Johnson et al. (2013a)
	68.8	6.6	–	–	–	7.8	Della Valle et al. (2013a)
CDC Richlea	56.3	6.8	69.7	27.1	18.8	–	Johnson et al. (2013a)
CDC Rosetown	62.3	6.8	62.3	24.8	19.0	–	Johnson et al. (2013a)
	71.8	6.5	–	–	–	8.4	Della Valle et al. (2013a)
CDC Rouleau	61.3	6.7	75.7	22.2	12.7	–	Johnson et al. (2013a)
	86.2	–	–	–	–	3.0	Della Valle et al. (2013b)
CDC Viceroy	61.3	7.7	76.6	24.4	18.6		Johnson et al. (2013a)
	74.4	–	–	–	–	3.5	Della Valle et al. (2013b)
Pennell	69.5	7.5	81.8	27.7	18	–	Johnson et al. (2013a)
Riveland	62.3	6.3	87.4	29.5	22.2	–	Johnson et al. (2013a)

Data from Johnson et al. (2013a), Della Valle et al. (2013a, b)

Overall, lentil cultivars Pennell and Rivel may have higher Fe bioavailability based on food matrix factors; however, the true Fe bioavailability has yet to be tested.

Iron is divided into two categories from a nutritional point of view: heme and nonheme. The heme form absorbs as a stable porphyrin complex that is unaffected by antinutrients including phytic acid, tannins, and phenolics. However, nonheme Fe from plant-based diets is easily transformed by antinutrient compounds. For example, antinutrients are capable of binding nonheme Fe and producing insoluble Fe complexes in the intestinal lumen, resulting in inhibition of Fe absorption. Two Fe-binding proteins occur naturally in foods—lactoferrin (animal foods) and phytoferritin (plant foods)—in which Fe is separated from chelates by a protein coat and made less sensitive to antinutrient factors present in the food matrix. Legume phytoferritin contains 1,800–2,200 atoms of Fe per molecule depending on the growing location and environment (Zhao 2010). Therefore, delivering considerable amounts of bioavailable Fe is possible by enhancing the phytoferritin levels in food legumes.

15.4.2 Lentil Prebiotics

Prebiotics are an important nutritional component of foods. The concept of prebiotics is based on dynamic processes of the microbiota in the intestinal tract, which comprises over 1,000 known species. These commensal microbes can be manipulated to benefit (or worsen) human health via the substrates that are made available for fermentation (undigested food components). A prebiotic is selectively fermented when consumed, altering the composition and/or activity of the microbiota to yield health benefits to the human host. A variety of health benefits may be provided from prebiotics, including immunostimulation (Guigoz et al. 2002), prevention of intestinal infections (Bosscher et al. 2006), increased bioavailability of minerals (Mg, Ca, Fe, and others) (Franck 2006), and reduced risk of osteoporosis (Abrams et al. 2005) and metabolic diseases.

Many carbohydrates that are now considered prebiotics were documented in lentil decades ago; however, discoveries of putative health benefits have cast their presence in a new light. Some prebiotic carbohydrates, such as raffinose family oligosaccharides (RFO) and resistant starch (RS), had been viewed as antinutrients. Breeders and food processors sought to remove RFO due to its implication in flatulence (Frias et al. 1999) and RS to enhance the available energy from food (Vidal-Valverde and Frias 1992). However, researchers have now begun to see prebiotic carbohydrates as a breeding trait not only for potential plant health and protection (Peters et al. 2007) but also for human health (Huynh et al. 2008).

In addition to RFO and RS, prebiotic carbohydrates in lentil include sugar alcohols and small quantities of fructooligosaccharides (FOS) (Bhatty 1988; Wang et al. 2009; Biesiekierski et al. 2011; Johnson et al. 2013b). Variation in seed RFO concentrations ranges from 1.8 to 7.5 % (Martinez-Villaluenga et al. 2008). Results of a 2-year replicated field study reveal genetic variability across ten commercial lentil genotypes (Johnson et al. 2013b). This variation is also observed in concentrations of two sugar alcohols, sorbitol (1,036–1,349 mg/100 g) and mannitol (158–294 mg/100 g), across genotypes (Johnson et al. 2013b). The varietal range in RS concentration in cooked lentil ranges varies from 3.7 to 5.1 g/100 g dry matter (Wang et al. 2009). FOS compounds nystose and kestose have been reported in lentil but at low concentrations and without significant genetic variation (Biesiekierski et al. 2011; Johnson et al. 2013b). A common finding accompanying genetic variation of expression of prebiotic carbohydrates is the influence of environmental factors on their concentrations in lentil seeds (Tahir et al. 2011; Johnson et al. 2013b). While the literature clearly suggests that the accumulation of these compounds in lentil can be manipulated through breeding efforts and by adapting plants to diverse locations, the potential effects of doing so have not been studied.

Prior to the development of effective breeding strategies to optimize prebiotic concentrations in lentil, a thorough understanding of changes that these compounds undergo during post-harvest storage, processing, and cooking procedures is required. RS and RFO (and FOS and sugar alcohols; unpublished data) all accumulate primarily in the cotyledon (Wang et al. 2009). The traditional consumption of lentil, either as a whole seed or dehulled and split, may have nutritional importance. Removal of the seed coat will concentrate prebiotic carbohydrates in the prepared food. Moreover, the fraction of starch that is resistant to human enzymes is highly sensitive to preparation procedures (Hoover and Zhou 2003). Cooling of boiled lentils nearly doubles the RS concentration, a phenomenon that is not reversed with reheating (Fig. 15.2).

15.4.3 Lentil Folates

Folate is another important micronutrient with respect to the prevention of preterm delivery, low birth weight, fetal growth retardation, and developmental neural tube defects (NTDs). Folate fortification and supplementation approaches have been attempted, but conflicting results regarding the ability to prevent NTDs and to increase the folate status at population levels in different countries have raised doubts about the continuous use of folic acid. The inability of folic acid to prevent NTDs and safety concerns with respect to too much folate in vulnerable population groups (e.g., children) demands alternative approaches to supply daily folates. Biofortification of staple crops with highly bioavailable folates might be a solution to folate deficiency. Lentils are naturally rich in folates, with levels in selected Canadian- and US-grown commercial lentil genotypes, quantified as folic acid equivalents, ranging from 275 to 622 μg/100 g (data not shown).

A recent study indicates that there is potential for genetic biofortification of lentils with bioavailable folates, quantified as tetrahydrofolate (Sen Gupta et al. 2013). Folate concentration in ten commercial lentil cultivars grown in North Dakota, USA, ranged from 216 to 290 μg/100 g,

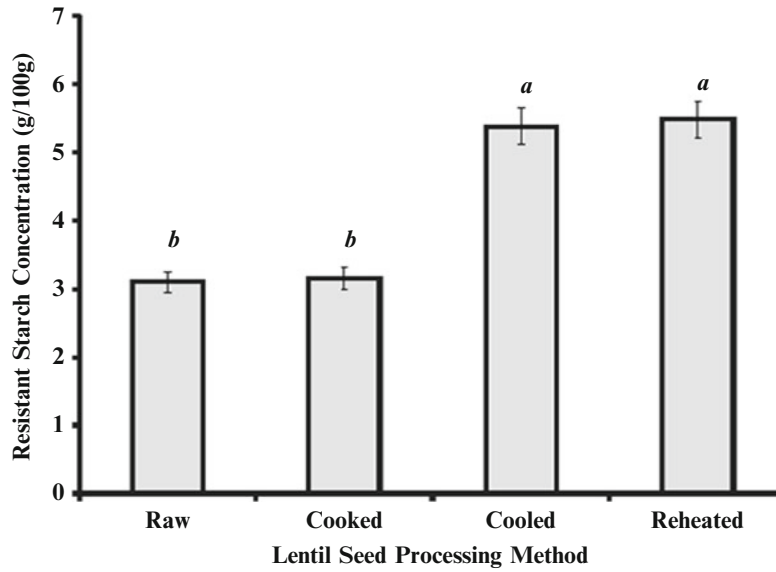

Fig. 15.2 Mean resistant starch concentration in raw, cooked, cooled, and reheated lentil products by whole and split market classes. Error bars are based on $p < 0.05$ ($n = 96$)

which would provide 54–73 % of the recommended daily allowance of dietary folate for adults (Sen Gupta et al. 2013). In addition, lentils have a higher folate concentration than chickpeas (42–125 μg/100 g), yellow field peas (41–55 μg/100 g), and green field peas (50–202 μg/100 g) (Sen Gupta et al. 2013). Further increases in folate levels and, more specifically, increases in bioavailable folate forms are possible through accurate identification and selection of genetic material and location sourcing. Overall, this limited set of data shows that a significant genetic effect could be further enhanced by careful selection of growing location (Sen Gupta et al. 2013).

15.5 Phenotyping Method for Micronutrient Analysis

15.5.1 Minerals

Mineral profiles of plant seeds can be analyzed by previously described modified HNO_3-H_2O_2 method (Thavarajah et al. 2009a). Finely ground seed sample (500 mg) digestion with nitric acid (70 % HNO_3) and hydrogen peroxide (30 %) leads to release of mineral micronutrients to the digested solution. The minerals in the digested solution can be carried out by atomic absorption spectroscopy or inductively coupled plasma emission spectroscopy.

15.5.2 Phytic Acid (PA)

PA involves prior PA extraction from sample prior to analysis (Thavarajah et al. 2009b). Simply, 0.5 M hydrochloric acid (HCl) addition to finely powdered sample and then heating in a boiling water bath enable PA extraction. The extracted PA could be decomplexed by addition of stronger acid (12.0 M HCl). For accurate PA identification and quantification, high-performance anion exchange (HPAE) with a conductivity detector was found to be a better method. In this procedure, an OmniPac Pax-100 anion exchange column with an OmniPac Pax-100 (8 μm) guard column (Dionex, Sunnyvale, CA, USA) with gradient mobile phase consisting of sodium hydroxide, deionized water-isopropanol, and water provide very good resolution of PA from other phytates. However, this method needs a strong anion suppressor because of higher conductivities of alkaline mobile phase.

15.5.3 Phenolics

Extraction and quantification of phenolic compounds could be carried out by the previously described method (Duenas et al. 2002). Phenolic compounds were extracted using methanol/water/acetic acid. The extracted phenolic can be analyzed by high-performance liquid chromatography (HPLC) system with photodiode array detection.

15.5.4 Folates

A finely ground sample needs to be dispersed in an extraction buffer consisting of potassium phosphate with sodium ascorbate and 2-mercaptoethanol. For foliate analysis, tri-enzyme treatment method is used. In this method, the homogenized seed samples need to be treated with enzymes -amylase, protease, and conjugase to release the folates from sample matrixes. The extracted folates can be analyzed on RP-HPLC (reversed-phase high-performance liquid chromatography) with fluorescent or mass detectors. This method has been described earlier (Sen Gupta et al. 2013).

15.6 Future Direction on Nutritional and Antinutritional Phenotyping

Both caloric and micronutrient malnutrition are important health concerns in developing countries. A lack of micronutrients in daily diets is a cause of many of the devastating health impacts felt by billions of people worldwide. Food systems based on a calorie-focused approach need urgent changes to control both malnutrition and associated diseases.

Past approaches to prevent micronutrient deficiency, including fortification, supplementation, and dietary diversification, have been met with limited success. However, biofortification of crops with micronutrients appears to be a sustainable solution to reduce micronutrient deficiencies. As such crops are developed and disseminated and become available to common consumers, biofortified foods could become a source of daily micronutrient requirements. To this end, pulse crops provide great promise. In particular, lentil is a protein-rich, medium-energy crop with a range of micronutrients. It has a high iron concentration with low levels of phytic acid and other iron bioavailability inhibitors and, hence, greater iron bioavailability. In addition, it is a rich source of prebiotics including fructose family oligosaccharides and resistant starches. While lentil may be an ideal crop for increasing bioavailable iron content, it is also a medium-energy food source that would serve to also reduce caloric malnutrition. Recent research clearly shows that certain lentil varieties have superior nutrient profiles, providing further opportunities for increased biofortification and bioavailability efforts. To seize these opportunities, extensive characterization of nutritional and anti-nutritional traits in different cultivars, including wild genotypes, may be required. This would be a fundamental step toward a systematic and cost-effective way to advance biofortification/bioavailability efforts.

Lentil is a pulse crop with several advantages: superior iron and prebiotic carbohydrate profiles, short cooking time (~10–20 min), and nitrogen-fixing abilities. Therefore, incorporation of lentil into future food systems may provide benefits beyond a food-based solution to malnutrition. Other crops, including other pulse crops, also need to be studied to provide greater diversity to food systems and consumer choices. Thus, intensive phenotypic characterization is essential. Resource allocation and shifting of "calorie"-focused thinking are urgently needed toward the development of food systems that will have sustainable human and environmental health benefits.

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Experimental Designs for Precision in Phenotyping

16

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Abstract

Precision phenotyping is the evaluation of a genotype's expression in a given environment with minimum influence of experimental error. This chapter presents the basic principles of experimental designs and lists commonly used experimental designs for phenotyping crop genotypes. Experimental designs include unreplicated designs, incomplete block designs, and variable replication block designs which can also be generated using some selected software. This chapter illustrates some of such experimental designs and key directives of the software which can be used to generate and analyze these designs have also been included.

Keywords

Phenotyping • Experimental designs • Statistical analysis

16.1 Introduction

Phenotyping stands for observing or evaluating a genotype in an environment, with least effect due to experimental error, while genotyping stands for observing and describing primarily the genetic makeup of the genotype which is done in terms of using various molecular markers such as amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), and single nucleotide polymorphism (SNP). A phenotype is an expression of the molecular construct of a genotype in a given environment and depends on the various sources that govern the expression. Thus, if a genotype is to be phenotypically evaluated in a specified factor-controlled/designated environment, for example, a drought-stressed environment, effort should be made to eliminate the effects of all other factors which influence the phenotypic expression. We will discuss designs commonly used for phenotyping in crop plants or for crop variety evaluation in general. The experimental designs may depend on the nature of genetic material and its availability.

The selection of the traits for phenotyping is important from various perspectives. Tuberossa (2011) has discussed key concepts, issues, and approaches for phenotyping for drought-stressed crops. The role of phenotyping of drought-adaptive traits and use of germplasm resources

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and genomic methods have been emphasized to improve drought resistance, and important elements of field plot techniques for obtaining phenotypic data under water-limited conditions have been listed. Recent interests have been found in identifying traits that can be used to identify genotype for climate change using climatic and agroecological information (Bari et al. 2012). The field-based precision phenotyping may be used to generate high-quality and large-scale datasets under managed stressed environments providing valuable guidance for drought screening (Campos et al. 2011). Depending on the trait, the mechanism of phenotyping could vary. Phenotyping can take place in Petri dishes in a temperature-controlled room, pots in a greenhouse, or plots in a field at a location with known biotic, abiotic, and edaphic conditions/factors. The phenotypic expression of the traits of interest of an object being phenotyped, for example, the genetic material, requires the identification of the population of the responding units, for example, the field plots under an environment with known stress levels.

The objective of this chapter is to briefly discuss basic principles of experimental designs and provides examples of various experimental designs used in phenotyping the crop genotypes at various stages of plant growth. We give main features of statistical analysis of data generated using such designs. We also overview some main statistical software which are used to generate these designs.

16.2 Design of Experiments

Experimental design for phenotyping will depend on the experimental material and sources of variation therein which are likely to distort the genotypic value of the genotypes. Experimental design is a mechanism to generate scientific evidences for collecting statistically valid and reliable pieces of evidence on the phenotype of the underlying genotype and is guided by the level of variability within the experimental material and size and shape of the experimental unit (e.g., a pot in a greenhouse and a plot in a field), operational convenience, and cost. The experimental material may be seeds of a genotype kept in Petri dishes for studying dormancy and germination, seedlings grown in tubes for tolerance to salinity levels, and plants in pots kept in the greenhouse for studying their response to controlled application of stress – moisture stress, insect, disease infection, or field plots for yield and yield components evaluation.

An experimental unit is the smallest division of the experimental material to which a genotype is assigned recognizing the fact that any neighboring experimental units may be assigned to different genotypes. A set of all the experimental units form the experimental material. In simplest terms, an experimental design is an assignment of treatments to the experimental units and is implemented using the principles of randomization, replications, and local control of experimental error or reduction of errors with a view to obtain a valid and precise evaluation of the treatments under investigation. These three basic principles of experimental designs are also known as 3Rs of Sir R.A. Fisher (1990). Randomization is a random assignment of genotypes (treatments) to the experimental units. It is a key element for assigning validity to the information on phenotype and forms the basis for describing the phenotype using a statistical model. Replication, the number of experimental units assigned to a given genotype, is essential for estimating the experimental error or experimental error variance which is a variation arising from the responses of the same genotypes on homogeneous experimental units. In reality, experimental material is not homogenous; the effort is made to eliminate the effect of any systematic factor using proper field plot management techniques and/or by accounting for these systematic factors, which helps in reducing the experimental error variance. The experimental error variance also depends on the size and shape of the experimental units determined by the nature of the experimental material required for phenotyping and the treatments applied.

The precision of the treatment performance or effect depends on the variability in the experimental material and number of replications and

can be increased by reducing the error variability and/or by increasing the replication. When the error variability cannot be reduced further, the number of replications (r) can be chosen to achieve estimates with a given precision using the following commonly used expression

$$r = \frac{4\theta^2}{\varepsilon^2}$$

where θ is the coefficient of variation of the trait of interest for the population or the experimental material after eliminating the effects of every systematic factors, and ε is the relative absolute difference in the observed treatment mean from the r replications and the true treatment mean. The basics of the principles of experimental designs are described in standard texts by Federer (1955), Cochran and Cox (1957), Kempthorne (1983), Cox and Reid (2000), Mead et al. (2002), Hinkelmann and Kempthorne (2005, 2008) among others. A number of specific situation-related experimental designs are given in Hinkelmann (2012). We also refer to a checklist of questions, that experimenters are advised to answer, provided by Jeffers (1978).

There are primarily two types of effects assumed for the treatments (genotypes) which form the basis for developing the criterion for which the designs are constructed. Under genotype effects assumed as fixed, designs are developed by minimizing the average variance of estimated difference between effects of pairwise treatments, and the resulting designs are called A-optimal (Kiefer 1959). Under this setup, one evaluates the phenotypes in the form of best linear unbiased estimates (BLUEs). The crop variety trial process comprises of selecting a number of desired genotypes from a much larger number under evaluation, and therefore, the genotypes keep varying with time and the prediction of future performance of a genotype is needed. In this situation, genotypes are seen to have been randomly drawn from a population or a process resulting from a breeding strategy, and the genotype effects may more appropriately be assumed as random. Maximization of a genetic gain or heritability is the parameters of interest. These lead to developing experimental designs which could optimize for average variance of predicted difference between the best linear unbiased predictors (BLUPs) (Cullis et al. 2006).

At various stages during plant development, observations are recorded on the expressions or responses in the field books or in an electronic form using handheld or other electronic devices. The data are then subjected to transformation, e.g., yields recorded at plot basis are transformed to yield per hectare, before using them in statistical analysis.

16.2.1 Software for Generating Experimental Designs

There are several statistical packages such as GenStat (Payne 2011), SAS (SAS Institute Inc. 1989), CycDesigN (Whitaker et al. 2002), AGROBASE (Agronomix Software Inc. 1999), etc. that can be used to generate randomized plans. The design for partial replications can be generated using codes of DiGGer, an R-package (Coombes 2009).

16.3 Data Analysis Procedures

Statistical analysis is a procedure to draw inference on the genotypes by searching pattern in the phenotypic evidences and assessing the strength of the pattern relative to the noise arising from experimental errors. The power of evidence on the genotype effects can be enhanced by incorporating any features inherited in the experimental material at the design and analysis stages. The data or response values are generally modeled using the following representation:

Data or function of (Data) = Pattern (experimental factors, environmental patters, any other systematic feature in the experimental material) + random error.

The total variability in the data is then partitioned into that due to various components of the patterns and errors. The error variance, measured by error mean squares, is used to assess the significance or contribution of the components of interest in the pattern. Often we use analysis of variance (ANOVA) and estimate means with standard errors and perform multiple comparisons, and residual plot analysis is used to examine the validity of assumptions underlying the ANOVA. We will now discuss a number of commonly used experimental designs for phenotyping in a wide range of disciplines, such as plant breeding and genetics, physiology, pathology, and entomology.

16.4 Experimental Designs for Phenotyping of Crop Genotypes

For phenotyping of improved genetic material generated through collection-selection missions or crossing and for its evaluation in field conditions, experimental designs are needed for preliminary screening, advanced yield trials, multi-locational trials, international nurseries, etc. as described below. The necessary codes and steps for using GenStat menus and R-package DiGGer are given in the Appendix.

16.4.1 Preliminary Screening/ Unreplicated Trials

At the preliminary stage of genetic material development or the early generation testing, the number of genotypes is often quite large with limited seeds which are sufficient for only one or a few replications. Further, seeds of a number of genotypes, called checks with similar maturity level, are available in sufficient number for required number replications for evaluation of experimental errors. A number of experimental designs that are available include reinforced block designs (Das 1958) and augmented designs in one-way blocks (Federer 1961) and two-way blocks (Federer and Raghavarao 1975; Lin and Poshinsky 1983). A randomized plan for 45 unreplicated test genotypes in 9 incomplete blocks of size 8, comprising 3 checks and 5 test entries, is given in Table 16.1.

Table 16.1 Layout of an augmented design in blocks containing test entries numbered from 1 to 45 and check entries numbered from 46 to 48

Blocks	1	2	3	4	5	6	7	8	9
Plots									
1	47	23	41	43	14	36	25	21	22
2	46	35	26	48	47	13	48	48	1
3	9	47	46	47	16	4	47	3	5
4	28	46	33	15	31	47	12	47	47
5	48	48	47	29	48	48	32	24	48
6	38	19	6	46	34	46	46	46	44
7	7	45	48	17	46	20	11	18	46
8	39	10	30	37	2	40	27	8	42

The statistical analysis model accounts for the effects of incomplete blocks or row and column effects and genotype effects. Interest lies in estimates of adjusted means for the genotypes and their standard errors, along with the estimate of error variance, coefficient of variation (CV%), standard errors of comparisons of two test entries, test and check entries, and two check entries. The software that could be used includes GenStat (REML command), SAS (PROC MIXED), and ICARDA programs using GenStat software codes.

16.4.2 Advanced Yield Trials

Considerable research has been undertaken on developing experimental designs for situations in crop variety evaluations where sufficient seeds are available to conduct replicated trials. Designs with high efficiency are available for almost any number of genotypes to be evaluated in practice. Our experience indicates that the following types of designs are generally used, although these are not our recommendations.

16.4.2.1 Small Number of Genotypes ($V < 8$)

Often the experimental units within small-sized blocks can be expected to be homogeneous. For

Table 16.2 A randomized plan of a Latin square design in six genotypes numbered 1–6

Columns	1	2	3	4	5	6
Rows						
1	1	2	4	5	6	3
2	4	5	1	2	3	6
3	6	4	3	1	2	5
4	2	3	5	6	4	1
5	5	6	2	3	1	4
6	3	1	6	4	5	2

phenotyping a relatively small number of genotypes in trays or pots in a greenhouse or in plots in the field, one may use randomized complete block (RCB) designs with larger number of replications resulting into error degrees of freedom around 30. For controlling experimental error variation in two directions, for example, in the field, Latin square (LS) designs and Youden square designs are found suitable. In LS designs, the number of replications is equal to the number of genotypes, while in RCB designs, they can be chosen at will. An example is given in Table 16.2.

16.4.2.2 Moderate Number of Genotypes ($V \leq 15$)

While scope lies in having a better control of variability, with moderate number of genotypes, frequent use of randomized complete block (RCB) designs can be found with three or more replications. An example is given in Table 16.3.

16.4.2.3 Large Number of Genotypes ($V > 15$)

In field trials, the plot-to-plot variability within block increases with the size of the block. If a large number of genotypes are experimented using complete blocks, then plot-to-plot variability within the large-sized blocks could be perceived to be considerably high, and thus RCB design may not give precise estimates unless replications are increased at added cost. Experimentation in relatively smaller-sized blocks, i.e., use of an incomplete block design, seems to be a favorable alternative. Further, it is possible to find designs in incomplete blocks

Table 16.3 A randomized plan for a randomized complete block design in four replications and 12 genotypes numbered 1–12

Blocks	1	2	3	4
Plots				
1	3	11	7	3
2	2	2	12	8
3	11	12	4	2
4	4	3	2	7
5	9	7	9	10
6	1	6	10	12
7	7	4	6	1
8	12	1	3	6
9	10	9	11	9
10	5	5	5	11
11	6	8	8	5
12	8	10	1	4

such that if we rearrange the incomplete blocks in a way that the group of incomplete blocks placed physically together on the layout also form full replicates. Such designs are called resolvable block designs. An advantage of resolvable block design is that the effectiveness of incomplete blocks can be assessed in relation to complete blocks. Literature contains several classes of resolvable incomplete block designs: balanced incomplete block designs, square lattice designs, rectangular lattices, and α-designs (also called α-lattices), which are based on the structure of the number of genotypes, e.g., it may be a square number or a rectangular number. The α-designs (Patterson and Williams 1976) are available for almost every practical number of genotypes, with a small difference in block sizes, and suit most of the field configurations (see details below). The number of replications can also be chosen at will.

16.4.2.4 α-Designs: A Class of Resolvable Incomplete Block Designs

Patterson and Williams (1976) introduced a class of resolvable incomplete block designs for any number of varieties ($\boldsymbol{v}$) and block sizes ($\boldsymbol{k}$) such that $\boldsymbol{v}$ is a multiple of $\boldsymbol{k}$, i.e., $\boldsymbol{v} = \boldsymbol{k}\mathbf{s}$ where $\mathbf{s}$ is the number of incomplete blocks of the same size as $\boldsymbol{k}$. Thus the square lattices, rectangular lattices, and resolvable cyclic designs are the special

cases of α-designs. Construction of these designs requires knowledge of generation array, a combinatoric concept and the methods have been given by Patterson and Williams (1976), Patterson et al (1978), and John and Williams (1995). However, these can be obtained by using CycDesigN software (Whitaker et al. 2002) and GenStat (Payne 2011) for a number of genotypes less than 100. These computer-generated methods have shown to provide high efficiency factors within their comparable class of designs for a wide range of parameter values. There may also be situations where the number of genotypes is not a multiple of block sizes, i.e., $\boldsymbol{v} \neq \boldsymbol{ks}$. Suppose the number of treatments $\boldsymbol{v}$ is represented by $v = k_1 s_1 + k_2 s_2$; $\boldsymbol{k}_1$, $\boldsymbol{k}_2$ and $\boldsymbol{s}_1$, $\boldsymbol{s}_2$ being positive integers. Every replication has $\boldsymbol{s}_1$ blocks of size $\boldsymbol{k}_1$ each and $\boldsymbol{s}_2$ blocks of size $\boldsymbol{k}_2$ each. In such situations, it is possible to develop designs with two block sizes $\boldsymbol{k}_1$ and $\mathbf{k}_2$ where $\boldsymbol{k}_1$ and $\boldsymbol{k}_2$ are very close, say, they have a difference $|\boldsymbol{k}_1 - \boldsymbol{k}_2|$ equal to 1 or 2. The small difference in the block size may still support the homogeneity of experimental error variances within such blocks. For example, for evaluating $\boldsymbol{v} = 23$ genotypes, one may use $\boldsymbol{v} = 23 = 4 \times 5 + 3 \times 1 = \boldsymbol{k}_1 \times s_1 + \boldsymbol{k}_2 \times s_2$, thus using 5 blocks of size 4 and 1 block of size 3 in each replicate. Such designs are derived by omitting one or more varieties of the α-designs with $\boldsymbol{v} = \boldsymbol{ks}$. Two examples of α-designs are given in Tables 16.4 and 16.5. In the case of the designs in Table 16.5, the empty cell need not be retained or if required for keeping the planting machinery or any other logistics, then it could be filled by a filler check genotype.

Table 16.4 A randomized plan for an alpha design in 40 genotypes, incomplete blocks of size 5 and 3 replications

	Plots	1	2	3	4	5
Replicates	Blocks					
1	1	7	40	12	30	36
	2	39	34	33	29	1
	3	26	32	4	31	14
	4	9	2	21	20	10
	5	25	17	6	23	19
	6	24	8	11	3	22
	7	15	37	13	5	28
	8	16	35	38	18	27
2	1	36	38	11	5	32
	2	3	13	16	39	30
	3	40	31	6	20	1
	4	27	8	25	28	33
	5	17	9	26	34	37
	6	2	29	24	23	15
	7	21	35	7	19	14
	8	12	22	10	4	18
3	1	33	2	17	16	32
	2	9	40	27	15	14
	3	20	8	26	23	30
	4	35	12	24	31	34
	5	11	4	19	39	28
	6	25	29	13	36	10
	7	37	22	6	7	38
	8	21	3	1	18	5

16.4.2.5 Designs Eliminating Heterogeneity in Two Directions

When the direction of soil fertility is unknown or if variability exists in two perpendicular directions in the field, it is often helpful to use two-way blocks in the field to reduce the experimental error. There are several designs controlling variability in two directions. Some of the frequently discussed designs are row-columns (Pearce 1975), Youden squares (Youden 1940), lattice squares, (Yates 1940; Cochran and Cox 1957), lattice rectangles (Federer and Raktoe 1965), row-columnα-designs (John and Eccleston 1986), and incomplete block designs with nested rows and columns (Singh and Dey 1979).

In recent years, a more realistic approach has been suggested for searching experimental designs using a criterion which maximizes genetic gain due to selection (Kempton 1984). Another related criterion, minimizes average pairwise prediction error variance, is presented by Cullis et al. (2006). These designs were obtained for an early generation variety trials (EGVTs), called p-rep designs, which are alternative to the augmented designs in blocks (referred as grid plots). Simulation studies, based on 1,000 runs and 12 different combinations of genetic variance ratio and spatial autocorrelation parameters along rows and columns, have shown that p-rep designs resulted in higher genetic gain. In variety evaluation, a more practical situation shows that different sets

Table 16.5 A randomized plan for an alpha design in 29 genotypes, incomplete blocks of sizes 4 and 5 and 3 replications

	Plots	1	2	3	4	5
Replicates	Blocks					
1	1	17	15	18	5	29
	2	3	8	1	9	16
	3	28	12	#	22	7
	4	2	26	23	19	24
	5	11	21	27	4	25
	6	10	14	6	20	13
2	1	29	10	25	7	8
	2	#	21	15	1	26
	3	3	4	23	28	14
	4	5	22	19	20	9
	5	2	16	13	27	17
	6	12	6	11	18	24
3	1	16	6	15	25	22
	2	23	8	#	11	17
	3	18	26	20	7	4
	4	14	27	19	12	1
	5	28	13	9	29	24
	6	3	21	2	5	10

#the empty plot need not be retained or, if required, could be filled by a suitable filler check

of genotypes could have seeds available for varying replications. Further, in the field layout, the spatial variability exists and the plot errors are generally correlated (Singh et al. 2003). To generate experimental designs incorporating the need of variable replications and correlated errors, Coombes (2009) has developed an R-program package called DiGGeR. An example of p-rep design is in Table 16.6 for 20 test genotypes with no replications and 10 test genotypes with 2 replications and 3 check genotypes. To generate randomized plans for p-rep designs, DiGGeR package in R-language programs has been developed by Coombes (2009).

16.4.3 Multi-environment Trials

Multi-environment trials (MET), normally designed in replicated designs, e.g., RCB or α-design, are conducted over multi-locations and multi-years to obtain information on the variety responses to the environments and study the nature of the genotype × environment (G × E) interaction. Main objectives of METs are selection of varieties for high and stable yield and their adaptability to specific zones (clusters) of the environments. The number and variability due to the locations, years, and experimental error may be used to determine the number of replications per trial. However, for moderately large number of locations and years, two replications per trial have been found to be optimal (Kempthorne 1983). Several methods of analyses can been found in literature and in several review papers (Lin et al. 1986; Westcostt 1986; Smith et al. 2005). The methods for analysis of G × E interaction studies have been used based on extracted patterns in the form of multiplicative models for G × E interaction (Gauch 1988), multiplicative model for G + G × E interactions, and factor analytic representations of G × E interactions using fixed genotype effects and random environmental effects (Piepho 1997) as well as fixed environmental effects and random variety effects (Smith et al. 2001). Smith et al. (2005) have reviewed mixed models used in multi-environment variety trials. Singh et al. (1996) using information on genotype means and standard errors in multi-location trials assessed these varieties using indices measuring inter-site transferability of varieties. The combined analysis at plot levels used to be under similar designs and under the assumption of homogeneous error variances, primarily due to

Table 16.6 A randomized plan, on an 8 × 8 layout, of a p-rep design in 33 genotypes numbered 1–20 have no replications, 21–30 have two replications, and 31–33 are checks with 8 replications

Rows/columns	1	2	3	4	5	6	7	8
1	4	33	20	29	24	5	31	32
2	31	9	25	23	1	32	19	27
3	14	30	21	10	31	18	33	28
4	32	13	33	17	21	27	24	31
5	6	28	23	32	12	26	30	33
6	31	16	22	3	33	11	32	15
7	7	32	26	33	22	31	8	32
8	33	29	31	25	32	2	33	31

limitations of computational software, but in the recent years, much more complex models, can be fit at the plot-level data with complex structures of variance-covariance matrices using GenStat, ICARDA modules in GenStat, SAS, AGROBASE, and ASReml (Gilmour et al. 2009).

A large number of phenotyped data are obtained through the international nurseries, with specific purposes, which facilitate screening and evaluation of genetic material across a wide range of environments. Experimental designs such as RCB, α-designs, and augmented designs are used. Trials should have independent randomizations. CGIAR (Consultative Group for International Agricultural Research) centers use an ICIS (International Crop Information System) for generating randomizations and storage and retrieval of crop information in terms of genotype pedigree and response data.

Inheritance studies form a part of the genetics of the traits used in phenotyping through the use of specific mating designs such as complete/partial diallel crosses and line × tester to generate information on the gene actions controlling the traits in terms of genetic ratios, genetic variance, and its components (such as additive, dominance, and allelic interactions of various orders). Embedding of mating and environmental designs derived from incomplete crosses, and blocks are discussed and reviewed by Singh et al. (2012).

Appendix

Some key codes used in generating the experimental designs under various tables.

A.16.1 GenStat Code for Table 16.2 (Geno Stands for Genotypes)

```
AGLATIN [PRINT=design; ANALYSE=Yes]
  NROWS=6; NSQUARES=1;\
TREATMENTFACTORS=!p(Geno);
  ROWS=Rows; COLUMNS=Columns;
  SEED=27257
```

A.16.2 GenStat Code for Table 16.3 (Rep, Plots, and Geno Stand for Replications or Complete Blocks, Plots Within Block and Genotypes Respectively)

```
AGHIERARCHICAL [PRINT=design;
  ANALYSE=Yes;SEED=2534]\
BLOCKFACTORS=Rep,Plots;
  TREATMENTFACTORS=*,!p(Geno);
  LEVELS=4,12
```

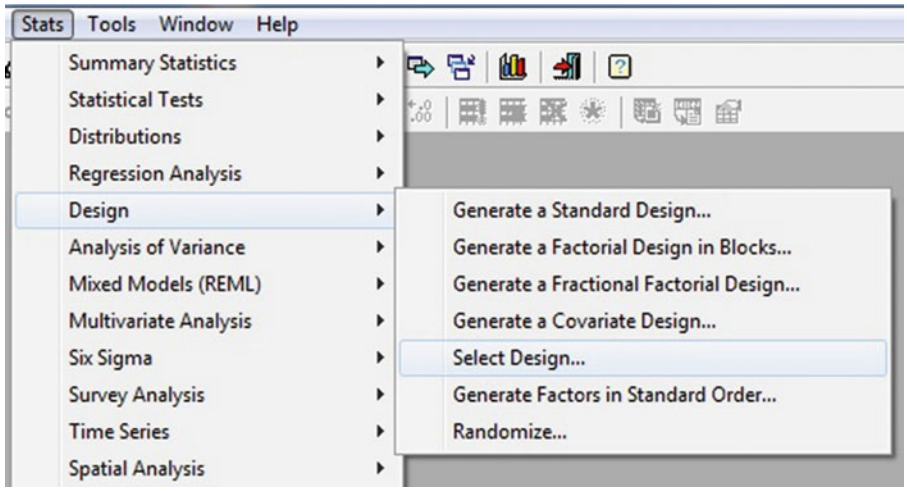
A.16.3 R Language Code for Table 16.6

```
library(DiGGer)
trep <- rep(c(1, 2, 8), c(20, 10, 3))
design <- DiGGer(33, 8, 8,
  TreatmentRep = trep)
design <- run(design)
getDesign(design)
layout <- getDesign(design)
des.plot(layout, seq(1, 20),
  col = 5, new = TRUE)
des.plot(layout, seq(21, 30),
  col = 6, new = FALSE)
des.plot(layout, seq(31, 33),
  col = 7, new = FALSE)
```

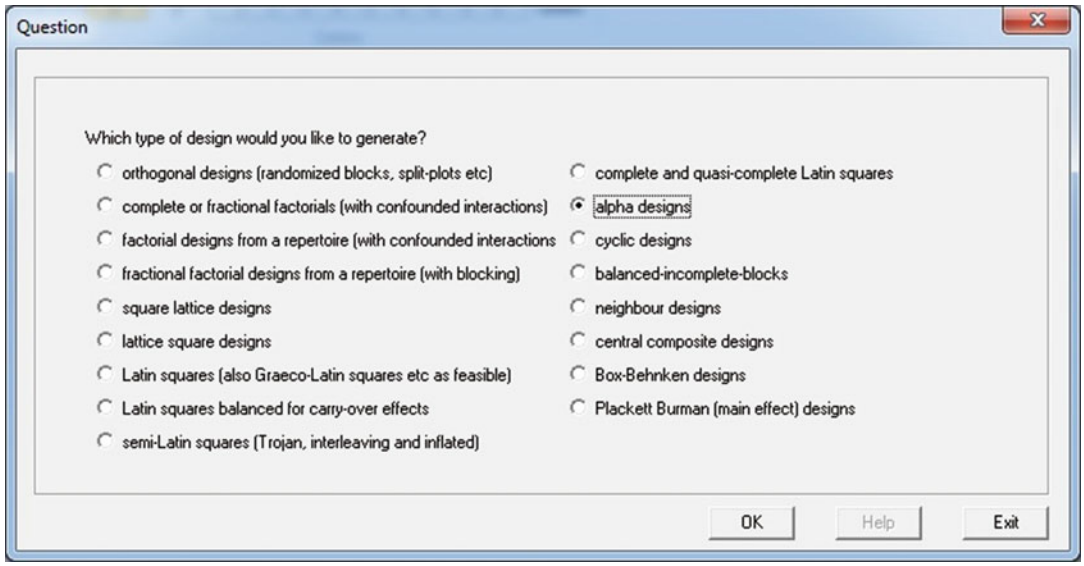
A.16.4 Further Details on GenStat Menu and R-Program

A.16.4.1 Generate an α-Design Using GenStat

To generate randomizations using GenStat statistical package (Payne 2011), go to its "Stats" menu, "Design" sub menu, and then "Select Design . . ." item (see the screenshot below):

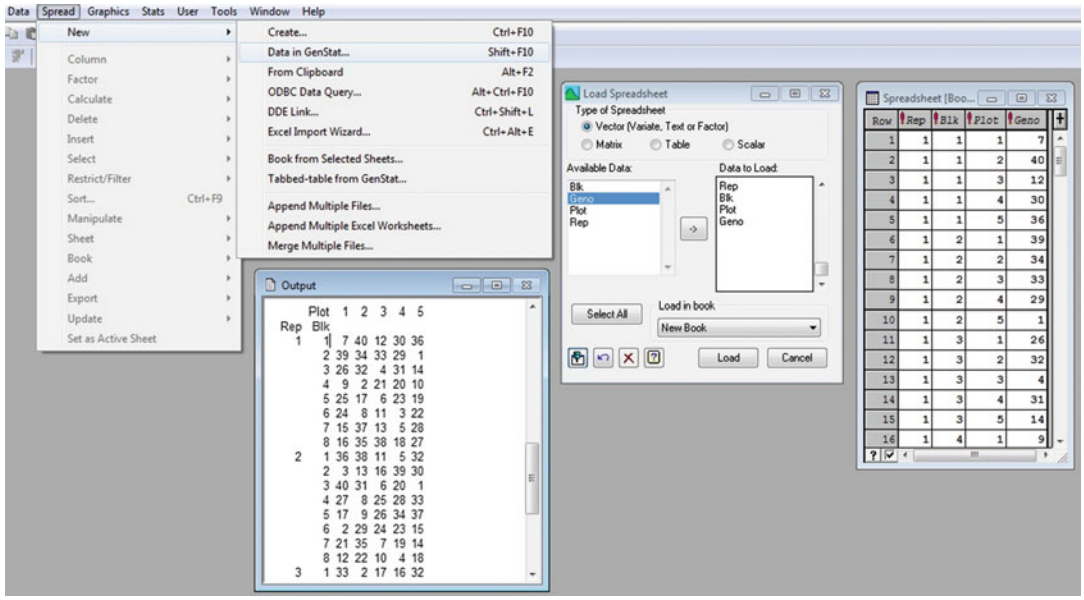


This will pop-up the dialog box listing several special analyses (see the screenshot below):



Select "alpha designs" option, then click "OK" button, and answer the series of questions on number of treatments (within the range 20–100), number of blocks per replication, number of replications, and the labels that should be assigned to the factors. Using the "Spread" menu and further "Data in GenStat" and item from "New" sub menu, one can obtain the randomized plan in the GenStat spreadsheet as shown in the following screenshot. For more than 100

genotypes, one may use CycDesigN software (Whitaker et al. 2002).



The plan in Table 16.4, in 40 genotypes in blocks of size 5 and 3 replications, can be obtained by running the following code:

```
AGALPHA [PRINT=design]
  LEVELS=40; NREPLICATES=3;
  NBLOCKS=8;\
    TREATMENTS=Geno;\
    REPLICATES=Rep;\
    BLOCKS=Blk;\
    UNITS=Plot;\
    SEED=1592654
```

A.16.4. 2R-Package DiGGer Codes for Table 16.6.

Generate Design for Partial Replications Using DiGGer and R Language:

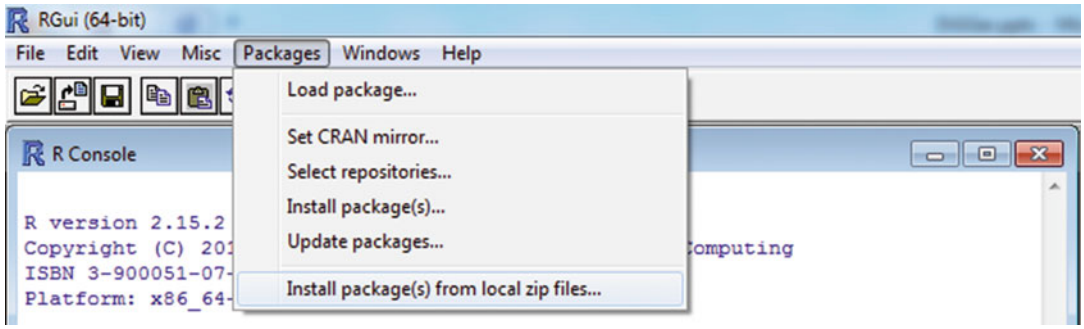
To use DiGGer tool, one needs to carry out required installation for the R package and download the following zip files "R. methodsS3_*.zip",[1] "R.oo_*.zip",[2] and "DiGGer_*.zip"[3] where "*" in the filenames denotes the current version available. Then one may start the R program, go to the "Packages" menu, and select "Install package(s) from local zip files. . .". Find the downloaded files and let R install them.

Once DiGGer packages are installed, the following codes are used to generate the experimental design in the Table 16.6.

[1] http://cran.rstudio.com/web/packages/R.methodsS3/index.html

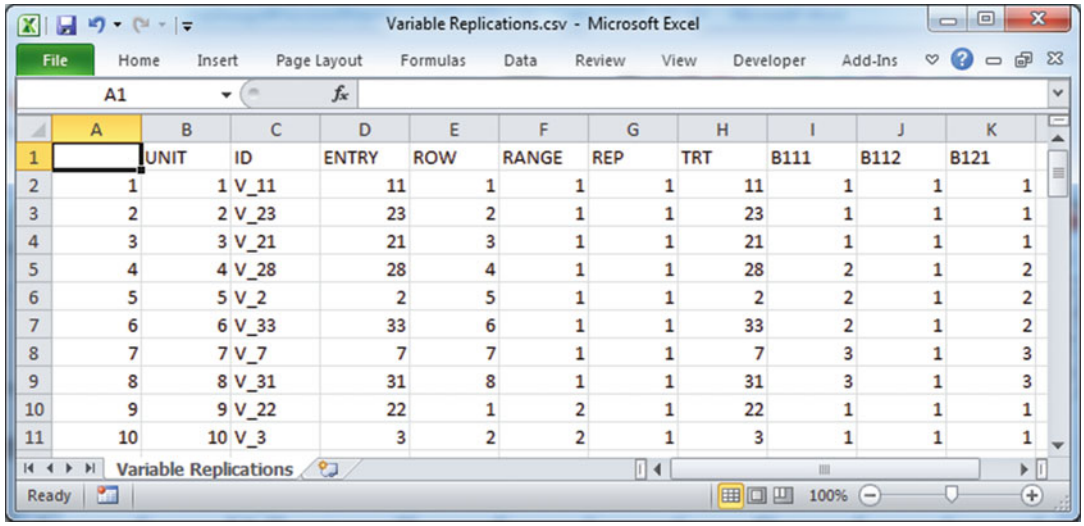
[2] http://cran.rstudio.com/web/packages/R.oo/index.html

[3] http://www.austatgen.org/files/software/downloads




```
# load required package
library(DiGGer)

# 20 genotypes with no replications [1 - 20]
# 10 genotypes with 3 replications [21 - 30]
# 3 genotypes with 8 replications [31 - 33]
trep <- rep(c(1, 2, 8), c(20, 10, 3))

# in total we have 33 genotypes (i.e. 20 + 10 + 3)
# in total we have 64 plots (i.e. 20*1 + 10*2 + 3*8)
# field layout set as 8 rows x 8 columns
design <- DiGGer(33, 8, 8, TreatmentRep = trep)

# once the design search object has been created
# we can produce the design
design <- run(design)

# extracting matrix of design numbers
layout <- getDesign(design)

# draw colored field layout
# or you may simply use plain plot(design) function in this case
des.plot(layout, seq(1, 20), col = 5, new = TRUE)
des.plot(layout, seq(21, 30), col = 6, new = FALSE)
des.plot(layout, seq(31, 33), col = 7, new = FALSE)

# export into CSV file
write.csv(design$dlist, "Variable Replications.csv")
```

Row \ Range	1	2	3	4	5	6	7	8
1	11	22	30	26	6	31	27	33
2	23	3	24	1	33	9	32	19
3	21	33	20	32	13	28	8	31
4	28	15	32	21	24	27	33	10
5	2	31	18	33	16	31	29	32
6	33	14	25	5	32	4	23	31
7	7	32	26	31	25	17	33	22
8	31	12	33	30	32	29	31	32



	A	B	C	D	E	F	G	H	I	J	K
1		UNIT	ID	ENTRY	ROW	RANGE	REP	TRT	B111	B112	B121
2	1	1	V_11	11	1	1	1	11	1	1	1
3	2	2	V_23	23	2	1	1	23	1	1	1
4	3	3	V_21	21	3	1	1	21	1	1	1
5	4	4	V_28	28	4	1	1	28	2	1	2
6	5	5	V_2	2	5	1	1	2	2	1	2
7	6	6	V_33	33	6	1	1	33	2	1	2
8	7	7	V_7	7	7	1	1	7	3	1	3
9	8	8	V_31	31	8	1	1	31	3	1	3
10	9	9	V_22	22	1	2	1	22	1	1	1
11	10	10	V_3	3	2	2	1	3	1	1	1

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Biometrical Approaches for Analysis of Phenotypic Data of Complex Traits

17

Huihui Li and Jiankang Wang

Abstract

Phenotype (or phenotypic value) is the performance of a trait in interest, which can be observed in the field and then used in estimating the unknown genotypic value (or the phenotypic mean). In this chapter, we introduced statistical approaches to analyze three types of phenotypic observation, i.e., (1) replicated observations of one genotype in one environment, (2) replicated observations of multiple genotypes in one environment, and (3) replicated observations of multiple genotypes in multiple environments. The principle of analysis of variance (ANOVA) was applied on each kind of phenotypic data. From the results of ANOVA, we can further estimate genotypic value, genetic effects, variance components, heritability, etc., which can be further used in genetic studies and breeding applications. In the end, we present a computer tool implemented in the integrated genetic software QTL IciMapping, which includes the biometrical approaches introduced in this chapter and can be readily used in phenotyping complex traits.

Keywords

Phenotype • Analysis of variance (ANOVA) • Genotype • Genetic variance • Heritability

17.1 Introduction

For making genetic improvement, plant breeders collect huge amount of phenotypic data on various populations. The phenotype of traits particularly quantitative traits is controlled by genotype and environments, and thus raw phenotypic data measured for various complex traits include the combined effect of both the genotypic value (G) and the environmental deviation (E): $P = G + E$. However, for making genetic improvement in trait, genotypic value is more important than the phenotypic value that is the combined effect of all the genetic effects, including nuclear genes, mitochondrial genes, and interactions

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between the genes. Therefore it is essential to know the contribution of heritable variation in total phenotypic variation of a quantitatively inherited trait. For this purpose, different statistical approaches have been used to study the inheritance of quantitative traits which is known as biometrical genetics. Therefore, this chapter has described the different approaches developed in biometrical genetics for analysis of phenotypic data for finding out the genotypic value of traits.

17.2 Basic Statistics Theory

17.2.1 Random Variable and Normal Distribution

If a random variable X has the following probability density, X is stated to have a normal distribution with mean μ and variance σ^2, where μ and σ^2 are known constants or unknown but estimable parameters:

$$f(X=x)=\frac{1}{\sqrt{2\pi\sigma^2}}e^{\frac{(x-\mu)^2}{2\sigma^2}} \tag{17.1}$$

The function given in Eq. (17.1) is called the probability density (PDF), and the random variable X is normally denoted by $X \sim N(\mu, \sigma^2)$. When the normal distribution has a mean of 0 and a variance of 1, the distribution is also called a standard normal distribution, denoted by $N(0, 1)$. For any normal distribution, $X \sim N(\mu, \sigma^2)$, $(X\text{-}\mu)/\sigma \sim N(0, 1)$, where σ is the square root of variance σ^2.

Assuming a random variable X has the PDF $f(x)$ and the possible value of X is any real number, mean (also called expectation) and variance of the random variable X are defined as follows:

$$E(X)=\int_{-\infty}^{+\infty} x\ f(x)dx, \tag{17.2}$$

$$V(X)=\int_{-\infty}^{+\infty}[x-E(x)]^2 f(x)dx = \int_{-\infty}^{+\infty}x^2 f(x)dx-[E(x)]^2 \tag{17.3}$$

The two statistics defined in the above two equations are also called mean and variance of the distribution of the random variable X. For a normal distribution $X \sim N(\mu, \sigma^2)$, it can be proved that $E(X) = \mu$ and $V(X) = \sigma^2$. This is the reason why the normal distribution $N(\mu, \sigma^2)$ is stated to have a mean of μ and a variance of σ^2. Or equally, if $X \sim N(\mu, \sigma^2)$, the random variable X is stated to have a mean of μ and a variance of σ^2.

17.2.2 Distributions Derived from the Standard Normal Distribution

From the standard normal distribution, we can define other distributions commonly used in statistical inference. If random variables $X_1, X_2, \ldots, X_n$ are independent and identical to the standard normal distribution $N(0, 1)$, the sum square of these variables is defined to follow a χ^2(chi--square) distribution with the degree of freedom of n, i.e.,

$$Y=X_1^2+X_2^2+\cdots X_n^2 \sim \chi^2(n) \tag{17.4}$$

Random variable $X \sim N(0, 1)$, random variable $Y \sim \chi^2(n)$, and the two variables are independent, and then $\frac{X}{\sqrt{\frac{1}{n}Y}}$ is defined to follow a t distribution with the degree of freedom of n, i.e.,

$$\frac{X}{\sqrt{\frac{1}{n}Y}} \sim t(n) \tag{17.5}$$

Figure 17.1 shows PDFs of the standard normal distribution $N(0, 1)$ and some t and χ^2 distributions. As $N(0, 1)$, t distribution is symmetrical. But, t distribution has much longer tails compared with $N(0, 1)$. With the increase in the degree of freedom, t distribution can quickly approach to the standard normal distribution (left of Fig. 17.1). In practice, when the degree of freedom is greater than 30, t distribution will be viewed to be the standard normal distribution. χ^2 distributions can only have positive values, by definition. With the increase in the degree of freedom, χ^2 distribution becomes much flatter

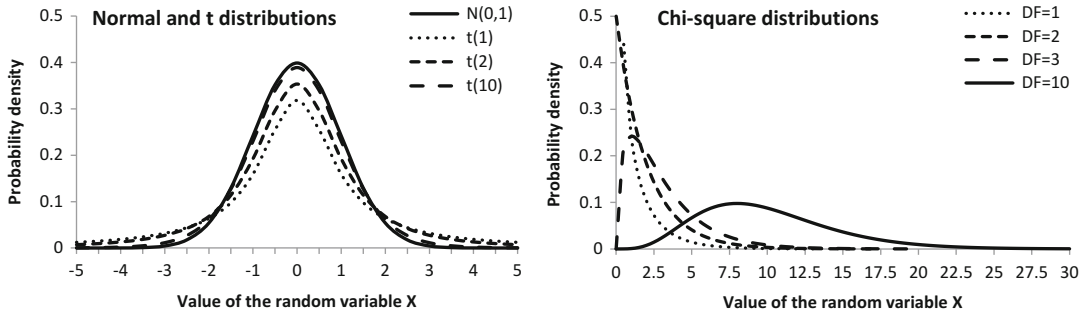


Fig. 17.1 Probability density functions (PDF) of the standard normal distribution $N(0, 1)$ and some t and χ^2 distributions

and has much longer tail to the right side (right of Fig. 17.1).

Assuming that two random variables $Y_1 \sim \chi^2(n_1)$ and $Y_2 \sim \chi^2(n_2)$ are independent, $\frac{\frac{1}{n_1}Y_1}{\frac{1}{n_2}Y_2}$ is defined to follow an F distribution with the two degrees of freedom of n_1 and n_2, i.e.,

$$\frac{\frac{1}{n_1}Y_1}{\frac{1}{n_2}Y_2} \sim F(n_1, n_2) \tag{17.6}$$

17.3 One Genotype in One Environment

17.3.1 Mean and Variance of a Phenotypic Distribution of Trait in Interest

It is assumed that we can repeatedly observe the phenotype (P) of a given genotype for a trait in interest in a given environment, and each observation is independent. True genotypic value in the environment is represented by G, which is an unknown parameter. Error in phenotypic measurement is a normally distributed random variable, having a mean of 0 and a variance of ${\sigma_\varepsilon}^2$. Error variance is unknown as well or has been estimated from previous experiments. Therefore, the observation will be independently and normally distributed around the true genotypic value G, and variance of the phenotypic distribution will be equal to the error variance ${\sigma_\varepsilon}^2$ in the environment. In statistics, we say these phenotypic observations have independently identical distributions (iid), that is,

$$P_k \sim N\left(G, \sigma_\varepsilon^2\right), \ (k = 1, 2, \ldots, r \text{ for replication}), \text{ iid} \tag{17.7}$$

In practice, we do not know the true genotypic value G and the true error variance ${\sigma_\varepsilon}^2$. However, they can be estimated from the replicated phenotypic observations P_k ($k = 1, 2, \ldots, r$), as the observation contains the information about these true values. This can be seen more clearly when distribution model (17.7) is represented in the following equivalent linear model:

$$\begin{aligned} P_k &= G + \varepsilon_k, \varepsilon_k \\ &\sim N\left(0, \sigma_\varepsilon^2\right), \ (k = 1, 2, \ldots, r), \text{ iid} \end{aligned} \tag{17.8}$$

Using the replicated observations, we can calculate the sample mean (represented by $\overline{P}$) and sample variance (represented by MS_ε) and use them as the estimates of the unknown G and error variance, respectively. Sample mean and the estimate of the genotypic value and their distributions are

$$\begin{aligned} \hat{G} &= \overline{P} = \frac{1}{r}\sum_k P_k, \text{ and } \hat{G} = \overline{P} \\ &\sim N\left(G, \ \frac{\sigma_\varepsilon^2}{r}\right) \end{aligned} \tag{17.9}$$

So, expectation of the estimated effect $\hat{G}$ is equal to the true genotypic effect. In statistics, we say the sample mean is an unbiased estimate of the

true genotypic effect. Variance of the estimate $\hat{G}$ is $\frac{1}{r}$ of the error variance. So, more observations give more precise estimate of the true effect. With the increase in sample size, the sample mean will asymptotically approach to the true genotypic value G. For this reason, the true genotypic value G is also called phenotypic mean. In statistics, it can also be proved that, among all possible unbiased linear combinations of observations ($k = 1, 2, \ldots, r$), the estimate given in Eq. (17.9) has the least variance. So the sample mean given in Eq. (17.9) is also called the best linear unbiased estimate (BLUE) of the phenotypic mean.

Sample variance (represented by MS_ε) and the estimate of the error variance (represented by $\hat{\sigma}_\varepsilon^2$) are

$$\hat{\sigma}_\varepsilon^2 = \mathrm{MS}_\varepsilon = \frac{1}{r-1}\sum_k \left(P_k - \overline{P}\right)^2 \tag{17.10}$$

For each observation, $(P_k\text{-}\overline{P})$ is the deviation of the observation from the sample mean, which can be used to measure random error effect in the observation. The sum square (SS) of each deviation is represented by SS_ε, i.e.,

$$\mathrm{SS}_\varepsilon = \sum_k \left(P_k - \overline{P}\right)^2 \tag{17.11}$$

Under the assumptions in distribution model (17.7) or equally in linear model (17.8), we can prove the following relationship between the expectations of SS_ε and ${\sigma_\varepsilon}^2$:

$$\begin{aligned} E(\mathrm{SS}_\varepsilon) &= E\sum_k \left(P_k - \overline{P}\right)^2 = E\sum_k \left[(P_k - G) - \left(\overline{P} - G\right)\right]^2 \\ &= \sum_k E(P_k - G)^2 - 2E\left[\left(\overline{P} - G\right)\sum_k (P_k - G)\right] + rE\left(\overline{P} - G\right)^2 \\ &= \sum_k E(P_k - G)^2 - 2E\left[\left(\overline{P} - G\right)r\left(\overline{P} - G\right)\right] + rE\left(\overline{P} - G\right)^2 \\ &= \sum_k E(P_k - G)^2 - rE\left(\overline{P} - G\right)^2 \\ &= r\sigma_\varepsilon^2 - r\frac{\sigma_\varepsilon^2}{r} = (r-1)\sigma_\varepsilon^2 \end{aligned} \tag{17.12}$$

The coefficient $(r - 1)$ before error variance in Eq. (17.12) is called the degree of freedom of the error effects. From Eqs. (17.10) and (17.11), it can be easily seen that

$$\begin{aligned} \hat{\sigma}_\varepsilon^2 = \mathrm{MS}_\varepsilon &= \frac{\mathrm{SS}_\varepsilon}{r-1}, \quad E\left(\hat{\sigma}_\varepsilon^2\right) \\ &= E(\mathrm{MS}_\varepsilon) = \sigma_\varepsilon^2 \end{aligned} \tag{17.13}$$

Therefore, sample variance is an unbiased estimate of the unknown error variance.

17.3.2 An Example on Plant Height in Four Genetic Populations

Table 17.1 gives observations of plant height (cm) in two inbred lines A and B and their F_1 and F_2 populations. Using Eqs. (17.9) and (17.10), we can estimate that inbred A has the a mean height of 160 cm, inbred B of 103 cm, F_1 hybrid of 149 cm, and F_2 population of 140 cm. Mean plant height in F_1 or F_2 population is between the two inbred parents. The two parents and their F_1 have similar variance, but variance of F_2 population is much greater. The larger variance in F_2 indicates the presence of genetic variance in plant height. If we can assume random error effects are homogeneous in the three non-segregating populations, we can combine the three sum squares to have one estimate of the error variance, i.e.,

Table 17.1 Plant height (cm) in two inbred lines and their F_1 and F_2 population. There are 10, 10, 10, and 30 observations in the four genetic populations

Population	Individual plant height (cm)	Sample mean	DF	SS	MS (=sample variance)
Inbred A	155, 161, 150, 164, 165, 161, 160, 158, 166, 164	160.40	9	222.40	24.71
Inbred B	97, 109, 92, 103, 109, 104, 98, 106, 102, 110	103.00	9	314.00	34.89
F_1	156, 148, 140, 150, 148, 147, 146, 155, 148, 150	148.80	9	183.60	20.40
F_2	89, 157, 149, 169, 123, 158, 151, 83, 167, 154, 152, 167, 116, 146, 97, 147, 162, 159, 111, 143, 144, 124, 137, 156, 80, 169, 157, 152, 157, 116	140.00	29	20074.00	692.21

$$\begin{aligned} \mathrm{SS}_T &= \mathrm{SS}_{P_1} + \mathrm{SS}_{P_2} + \mathrm{SS}_{F_1} = 720, \\ \mathrm{DF}_T &= \mathrm{DF}_{P_1} + \mathrm{DF}_{P_2} + \mathrm{DF}_{F_1} \\ &= 27, \quad \hat{\sigma}_\varepsilon^2 = \frac{\mathrm{SS}_T}{\mathrm{DF}_T} = 26.67 \end{aligned} \tag{17.14}$$

Assuming the height of inbred B has the normal distribution $N(100, 30)$, based on the observed height in Table 17.1. Therefore, as a random variable, the height has a mean of 100 and a variance of 30. Figure 17.2 shows the distribution curves of sample means for several sample sizes. It is clear that larger sample size results in smaller variance in the sample mean. Each curve in Fig. 17.2 represents how the sample mean will be distributed if we can repeat the sampling procedure infinitely. In practice, we normally have one set of samples. In Table 17.1, we only have one set of 10 phenotypic height values. Therefore, that is no guarantee that the sample mean of inbred B (i.e., 103 cm) is equal to the true genotypic height.

The unbiased sample mean to the true value, as given in Eq. (17.9), is a statistical property from the large number of sampling. The same is true for the sample variance. In statistics, the unknown parameters can be estimated from samples drawn from their population. But this does not indicate that the estimated value will be equal to the unknown parameter. Instead, each sample drawn from a population in interest is viewed as a random variable. Any estimate from a set of samples is also a random variable. Unknown parameters are normally assumed to be constants. Therefore, it does not make sense to say that a random variable is equal to a constant.

But, to know the distribution of a sample, statistics is good enough for conducting statistical inference and test. Say, we can tell how likely the true height is located in a given interval, how likely the true height is different from another genotype or genetic population, and so on. For example, using Table 17.1, we can tell the probability that the true height of inbred B is from 95 to 105 cm. We can calculate the significance probability between inbred A and inbred B, where t distribution will be used. We can test whether the genetic variance of F_2 population is significant, where F distribution will be used.

In addition, if we can assume random errors in F_2 populations have equal variance as estimated in Eq. (17.14), we are able to estimate the genetic variance of F_2 by subtracting the error variance from the phenotypic variance. That is,

$$\hat{\sigma}_G^2 = \hat{\sigma}_P^2 - \hat{\sigma}_\varepsilon^2 = 692.21 - 26.67 = 665.54$$

And the heritability in the broad sense in the F_2 population can be estimated,

$$H = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_P^2} = \frac{665.54}{692.21} = 0.96$$

17.3.3 Calculating Sample Mean and Sample Variance from Frequency Data

In many cases, the raw data with large sample size are grouped and the frequency of each group is given instead. Table 17.2 shows the number of samples falling in each group represented by the mid-group value of ear length (cm) in four genetic populations (East 1911). Taking inbred

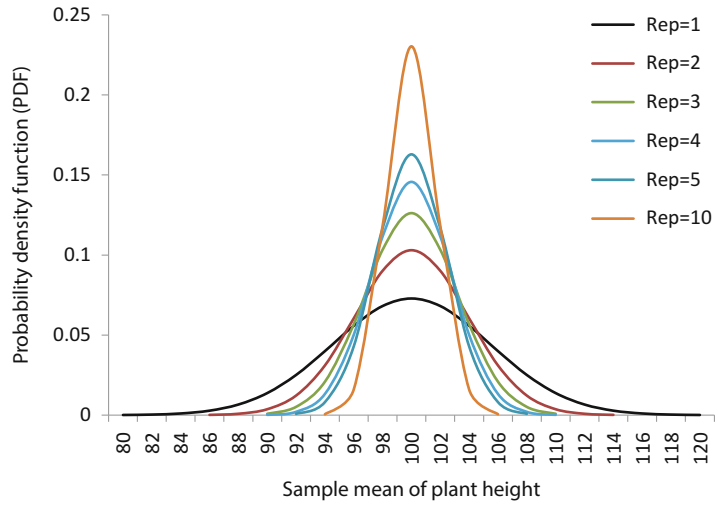


Fig. 17.2 Distribution of the sample mean of inbred B plant height. As a random variable, the height of inbred B is assumed to be normally distributed, having a mean of 100 and a variance of 30

Table 17.2 Frequency of ear length (cm) in four genetic populations

Ear length	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Size
Inbred A	4	21	24	8	–	–	–	–	–	–	–	–	–	–	–	–	–	57
Inbred B	–	–	–	–	–	–	–	–	3	11	12	15	26	15	10	7	2	101
F_1	–	–	–	–	1	12	12	14	17	9	4	–	–	–	–	–	–	69
F_2	–	–	4	5	22	56	80	145	129	91	63	27	17	6	1	–	–	646

Adapted from East (1911)

F_1 is the hybrid between the two inbred lines, and F_2 is the selfing generation of the F_1 hybrid

A as an example, among the 57 ears, 4 have ear length between 4.5 and 5.5, 21 have ear length between 5.5 and 6.5, 24 have ear length between 6.5 and 7.5, and 8 have ear length between 7.5 and 8.5. Let x_k be the mid-value of the kth group and $f_k = \frac{n_k}{n}$ be the relative frequency, estimates of the population mean and variance are, therefore,

$$\begin{aligned}\hat{\mu} &= \sum_k f_k x_k, \quad \text{and } \hat{\sigma}_\varepsilon^2 \\ &= \sum_k f_k x_k^2 - \hat{\mu}^2 \end{aligned} \tag{17.15}$$

One may find that the sample mean and variance given in Eq. (17.15) are similar to the distribution mean and variance given in Eqs. (17.2) and (17.3). From the above equation, means and variances of the four populations can be estimated. That is,

$$\overline{P}_A = 6.63, \ \overline{P}_B = 16.80, \ \overline{P}_{F_1} = 12.12, \ \overline{P}_{F_2} = 12.68$$
$$\hat{\sigma}_A^2 = 0.65, \ \hat{\sigma}_B^2 = 3.53, \ \hat{\sigma}_{F_1}^2 = 2.28, \ \hat{\sigma}_{F_2}^2 = 3.97$$

Assuming that errors have the same variance in these populations, we may use average of variances in the three non-segregating populations to estimate the true error variance and estimate the genetic variance and heritability in the F_2 population. That is,

$$\hat{\sigma}_\varepsilon^2 = 2.15, \ \hat{\sigma}_G^2 = \hat{\sigma}_{F_2}^2 - \hat{\sigma}_\varepsilon^2 = 1.82, \ H = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_{F_2}^2}$$
$$= \frac{1.82}{3.97} = 0.46$$

In addition, if the multifactorial hypothesis in classical quantitative genetics is applicable, we could estimate the number of loci (l) affecting ear length in these populations from the Castle-Wright formula. That is,

$$l = \frac{\left(\overline{P}_A - \overline{P}_B\right)^2}{8\left(\hat{\sigma}^2_{F_2} - \hat{\sigma}^2_{\varepsilon}\right)} \approx 7 \tag{17.16}$$

The multifactorial hypothesis mentioned above is fundamental in classical quantitative genetics. Major content in the hypothesis is that quantitative traits are controlled by a large number of Mendelian genes having smaller effects and can be easily modified by environments. In addition to the multifactorial hypothesis, when calculating the number of loci affecting ear length in Eq. (17.16), we also assume that the genes have equal additive effect on ear length, inbred A has all the alleles reducing the ear length, and inbred B has all the alleles increasing the ear length.

17.4 Multiple Genotypes in One Environment

17.4.1 Assumptions and Models

It is assumed that we make the field phenotyping experiment with r replications for a set of g genotypes in a given environment. The phenotypic means are represented by μ_i ($i = 1, 2, \ldots, g$), which are unknown parameters. Error effects are normally distributed with a mean of 0 and an unknown variance ${\sigma_\varepsilon}^2$. Randomization of genotypes in the field will assure that the observations are independent. So the observed phenotype for the ith genotype and kth replication is

$$P_{ik} \sim N\left(\mu_i, \sigma^2_\varepsilon\right), (i = 1, 2, \ldots, g;\ \ k = 1, 2, \ldots, r) \tag{17.17}$$

Therefore, we are having g normal distributions, corresponding to the g genotypes. The populations may have different means, but they should have the equal variance, which is actually the random error variance.

Given the phenotypic means of g genotypes, we can define an overall phenotypic mean, that is, $\mu \hat{=} \frac{1}{g}\sum_i \mu_i$. By defining the deviation of each phenotypic mean to the overall mean as the genotypic effect, represented by G_i, we can have the following linear model:

$$P_{ik} = \mu_i + \varepsilon_{ik} = \mu + G_i + \varepsilon_{ik},$$
$$\text{where } \mu \hat{=} \frac{1}{g}\sum_i \mu_i, \text{and } \varepsilon_{ik} \sim N\left(0,\ \sigma^2_\varepsilon\right)\ (i = 1,\ 2,\ \ldots, g; k = 1,\ 2,\ \ldots, r) \text{ and iid} \tag{17.18}$$

Genetic variance can be defined from the g phenotypic means:

$$\sigma^2_G \hat{=} \frac{1}{g-1}\sum_i G_i^2 \tag{17.19}$$

17.4.2 Estimation of Genotypic Effect and Genetic Variance

As we could see, there are two major purposes when making field experiment. The first one is to estimate the phenotypic means of a set of genotypes, as defined in distribution model (17.17) or in linear model (17.18). Based on the estimation of phenotypic means, we can conduct further genetic study, say QTL mapping. In the perspective of breeding, we can decide which genotypes have better performance and should be selected and advanced to the next season or which genotypes should be grown in this environment. The second one is to estimate error variance as defined in linear model (17.18) and genetic variance as defined in Eq. (17.19). From the estimation of the two variances, we can estimate heritability, which has been seen in the previous section.

Now we will show how the genotypic effects $G_i (i = 1,\ 2, \ldots, g)$, genetic variance ${\sigma_G}^2$, and error variance ${\sigma_\varepsilon}^2$ can be estimated by using observations P_{ik} ($i = 1, 2, \ldots, g$; $k = 1, 2, \ldots, r$). First, we define the overall sample mean ($\overline{P}..$), i.e., the mean across the g genotypes and the r replications. Second, we

define sample mean for each genotype ($\overline{P}_i.$), i.e., the mean across the r replications for each genotype. Based on the distribution model (17.17) of each observation, we have

$$\overline{P}.. = \frac{1}{gr}\sum_{j,k} P_{jk} \sim N\left(\mu, \frac{\sigma_\varepsilon^2}{gr}\right), \text{ and } \overline{P}_i. = \frac{1}{r}\sum_{k} P_{ik} \sim N\left(\mu_i, \frac{\sigma_\varepsilon^2}{r}\right)$$

By defining the overall mean and sample mean of each genotype, the phenotype can be decomposed as:

$$P_{ik} = \overline{P}.. + \left(\overline{P}_i. - \overline{P}..\right) + \left(P_{ik} - \overline{P}_i.\right) \text{ or equally} \quad (17.20)$$

$$P_{ik} - \overline{P}.. = \left(\overline{P}_i. - \overline{P}..\right) + \left(P_{ik} - \overline{P}_i.\right) \quad (17.21)$$

In Eq. (17.20), the first term is the overall sample mean. The second term is the deviation of the sample mean of each genotype to the overall mean. The third term is the residual deviation. The three terms in Eq. (17.18) can be used to estimate the three parameters defined in the distribution model (17.18). That is,

$$\hat{\mu} = \overline{P}.., \hat{G}_i = \left(\overline{P}_i. - \overline{P}..\right), \hat{\varepsilon}_{ik} = \left(P_{ik} - \overline{P}_i.\right) \quad (17.22)$$

Given the observed phenotypic values P_{ik} $(i = 1, 2, \ldots, g; k = 1, 2, \ldots, r)$, we can use Eq. (17.20) to estimate the overall mean, genotypic effect, and residual effect (if we want). To estimate genotypic variance and error variance, we have to consider the sum of the squared deviations. Total sum square (SS_T) is defined from the left side of Eq. (17.21). Total sum square can be further decomposed into two parts, which are represented by SS_G and SS_ε, corresponding to the two terms in the right side of Eq. (17.21). That is,

$$SS_T = \sum_{i,k}\left(\overline{P}_{ik} - \overline{P}..\right)^2 = \sum_{i,k}\left[\left(\overline{P}_{ik} - \overline{P}_i.\right) + \left(\overline{P}_i. - \overline{P}..\right)\right]^2 = \sum_{i,k}\left(\overline{P}_{ik} - \overline{P}_i.\right)^2 + r\sum_{i}\left(\overline{P}_i. - \overline{P}..\right)^2 = SS_\varepsilon + SS_G$$

We have a total of $g \times r$ independent observations. Total sum square (SS_T) has a degree of freedom of $gr - 1$. The one degree of freedom can be understood as being used in the estimation of the overall sample mean. Without the overall sample mean, we are unable to estimate the deviations on the left side of Eq. (17.21). Sum square of the estimated genotypic effects, i.e., SS_G, has a degree of freedom of $g - 1$. There are g estimated genotypic effects (Eq. 17.22), but the sum of these effects is equal to 0. So, the degree of freedom of $g - 1$ can be understood as the number of independent estimated genotypic effects. There are gr estimated residual effects (Eq. 17.20), but they are not completely independent. The degree of freedom of $g(r - 1)$ can also be understood as the number of independent estimated residual effects. Of course, it can also be found by subtracting $g - 1$ from the total degree of freedom $gr - 1$.

Mean square (MS) is defined as the sum square divided by its degree of freedom. That is,

$$MS_G = \frac{SS_G}{g - 1}, \text{ and } MS_\varepsilon = \frac{SS_\varepsilon}{g(r - 1)}$$

Intuitively, mean square of estimated genotypic effects reflects the magnitude of genotypic variance defined in Eq. (17.19). Mean square of the residual effects reflects the magnitude of error variance defined in distribution model Eq. (17.18). In statistics, we can prove

Table 17.3 ANOVA of single-environmental phenotyping trials of multiple genotypes

Source of variation	Degree of freedom (DF)	Sum square (SS)	Mean square (MS)	Expected mean square (EMS)
Genotype	$g-1$	SS_G	MS_G	$\sigma_\varepsilon^2 + r\sigma_G^2$
Error	$g(r-1)$	SS_ε	MS_ε	${\sigma_\varepsilon}^2$
Total	$gr-1$	SS_T		

$$E(SS_G) = (g-1)\sigma_\varepsilon^2 + (g-1)r\sigma_G^2,\ \ E(SS_\varepsilon) = g(r-1)\sigma_\varepsilon^2 \quad (17.23)$$

Therefore,

$$E(MS_G) = \sigma_\varepsilon^2 + r\sigma_G^2,\ \ E(MS_\varepsilon) = \sigma_\varepsilon^2 \quad (17.24)$$

From Eq. (17.24), we can see that the expectation of MS_ε is equal to the error variance and therefore is the unbiased estimate of error variance. In addition to genetic variance, error variance is also included in the expectation of MS_G. Therefore, $\frac{1}{r}MS_G$ cannot be an unbiased estimate for genotypic variance. Instead, we can have the following unbiased estimates for error variance and genotypic variance:

$$\hat{\sigma}_\varepsilon^2 = MS_\varepsilon,\ \ \hat{\sigma}_G^2 = \frac{1}{r}(MS_G - MS_\varepsilon) \quad (17.25)$$

The above procedure is called analysis of variance (ANOVA) in statistics and can be summarized in Table 17.3. An F-statistic can be constructed to test the significance of the genotypic variation compared with error, i.e.,

$$F = \frac{MS_G}{MS_\varepsilon} \sim F[g-1,\ g(r-1)] \quad (17.26)$$

In many cases, each replication of the g genotypes may be arranged in one relatively homogeneous block. Variation between blocks can occur. The use of block is another important concept in experimental design, which can reduce the random error variance and improve the precision when comparing genotypes. In this case, the deviation of the block mean to the overall sample mean estimates the block effect (represented by B_k), i.e.,

$$\hat{B}_k = (\overline{P}_{\cdot k} - \overline{P}_{\cdot\cdot}),\quad (k = 1,\ 2,\ \ldots, r) \quad (17.27)$$

And linear model (17.21) becomes

$$P_{ik} - \overline{P}_{\cdot\cdot} = (\overline{P}_{\cdot k} - \overline{P}_{\cdot\cdot}) + (\overline{P}_{i\cdot} - \overline{P}_{\cdot\cdot}) + (P_{ik} - \overline{P}_{i\cdot} - \overline{P}_{\cdot k} + \overline{P}_{\cdot\cdot}) \quad (17.28)$$

Similarly, ANOVA can be done based on the above model. It can be seen that including the block effect will not affect the estimation of genotypic effects and the genotypic variance but will affect the estimation of residual effects and the error variance. When the block effect is significant, estimated error variance will be lower than that from linear model (17.21). The reduced error variance allows more precise comparison of phenotypic means. In practice, other options are to estimate the block effect using Eq. (17.27), adjust the raw data by the block effect, and apply linear model (17.21) on the adjusted phenotypic observations.

17.4.3 Estimation of Heritability in the Broad Sense

As represented by the linear model (17.18), in single environment, phenotype of a quantitative trait for a given genotype or line or family can be decomposed into three parts: (1) overall mean across genotypes and replications, (2) genotypic effect of the specific genotype, and (3) random residual error. That is,

$$P = \mu + G + \varepsilon \quad (17.29)$$

where overall mean and genotypic effect are assumed to be unknown parameters, and residual error is assumed to be random variable. When residual error has a normal distribution, the phenotypic variance ${\sigma_P}^2$ is equal to the sum of genotypic variance ${\sigma_G}^2$ and error variance ${\sigma_\varepsilon}^2$. In session 3.2, we have seen that ANOVA can acquire the unbiased estimates of genotypic variance and error variance. Therefore, we can have

Table 17.4 Yield performance of ten maize inbred lines in three replications

Genotype	Replication			Phenotypic mean $(\overline{P}_{i\cdot})$	Estimated genotypic effect $(\hat{G}_i)$
	I	II	III		
RIL1	2.56	2.66	2.43	2.550	−0.247
RIL2	2.66	2.50	2.75	2.637	−0.160
RIL3	2.93	2.97	2.70	2.867	0.070
RIL4	2.57	2.21	1.80	2.193	−0.604
RIL5	3.06	2.61	2.72	2.797	0.000
RIL6	1.94	2.16	2.14	2.080	−0.717
RIL7	1.85	1.69	2.25	1.930	−0.867
RIL8	3.83	3.58	3.80	3.737	0.940
RIL9	4.32	4.12	4.14	4.193	1.396
RIL10	2.81	3.33	2.81	2.983	0.186
Block mean $(\overline{P}_{\cdot k})$	2.853	2.783	2.754	$\overline{P}_{ik} = 2.797$	
Estimated block effect $(\hat{B}_k)$	0.056	−0.014	−0.043		

the unbiased estimate of phenotypic variance as follows:

$$\sigma_P^2 = \sigma_G^2 + \sigma_\varepsilon^2 \tag{17.30}$$

In quantitative genetics, proportion of genetic variance over phenotypic variance is defined as the heritability in the broad sense, represented by H^2, i.e.,

$$H^2 = \frac{\sigma_G^2}{\sigma_P^2} = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_\varepsilon^2} \tag{17.31}$$

Therefore, applying the estimates of genotypic and error variances in Eq. (17.31), we can estimate the heritability in the broad sense of quantitative traits.

The heritability estimated by the formula (17.31) is based on single observations. In many cases, genetic analysis is based on phenotypic mean across replications, i.e., $\overline{P}_{i\cdot}$. In this case, we may want to estimate the heritability based on $\overline{P}_{i\cdot}$, and Eqs. (17.29), (17.30), and (17.31) become

$$\overline{P} = \mu + G + \overline{\varepsilon} \tag{17.32}$$

$$\sigma_{\overline{P}}^2 = \sigma_G^2 + \frac{1}{r}\sigma_\varepsilon^2 \tag{17.33}$$

$$H^2 = \frac{\sigma_G^2}{\sigma_{\overline{P}}^2} = \frac{\sigma_G^2}{\sigma_G^2 + \frac{1}{r}\sigma_\varepsilon^2} \tag{17.34}$$

Obviously, a higher heritability will be achieved, when phenotypic mean is used.

17.4.4 An Example on Yield of Ten Maize Inbred Lines in One Environment

To investigate the yield performance of ten maize inbred lines, randomized block design (RBD) is used, where each replication of the ten lines is arranged in one homogenous field block. Yield is measured on each plot, and raw data is given in Table 17.4. By raw data, we first calculate the mean for each row and mean for each column. The mean for each row is the phenotypic mean of the three replications of each inbred, and the mean for each column is the block mean. The result is respectively given in column 5 and row 13 in Table 17.4. Then we calculate the overall mean, i.e., $\overline{P}_{ik} = 2.80$, in Table 17.4. Finally we can calculate the genotypic effect and the block effect. Genotypic effect is the deviation of the phenotypic mean to the overall mean, and block effect is the deviation of the block mean to the overall mean. The result is respectively given in column 6 and row 14 in Table 17.4.

From the estimated block effect in the last row in Table 17.4, we can see that block effect may not be important. If we can ignore the block effect and use linear model (17.20), the ANOVA result is given in Table 17.5. It can be seen that the ten inbred lines show significant difference on yield. From the two mean squares, the error variance is estimated at 0.0473, and the genotypic variance is estimated at 0.4941. From

Table 17.5 Yield performance of ten maize inbred lines in three replications

Source	DF	SS	MS	Estimated variance	*F* value	*P* value
Genotype	9	13.768	1.530	0.494	32.346	0.000
Error	20	0.946	0.047	0.047		
Total	29	14.714				
R^2 (%)	93.571					
H^2 per plot	0.913					
H^2 per mean	0.969					

estimated variances, heritability in the plot level is estimated at 91.27 %, and heritability in the phenotypic mean level is estimated at 96.91 %.

17.5 Multiple Genotypes in Multiple Environments

17.5.1 Assumptions and Models

It is assumed that we make the field phenotyping experiment with r replications for a set of g genotypes in a set of e environments. The phenotypic means of the g genotypes in e environments are represented by $\mu_{ij}(i = 1, 2, \ldots, g; j = 1, 2, \ldots, e)$, which are unknown parameters. Error effects are normally distributed with a mean of 0 and an unknown variance ${\sigma_\varepsilon}^2$. So the observed phenotype for the ith genotype, jth environment, and kth replication is

$$P_{ijk} \sim N\left(\mu_{ij},\ \sigma_\varepsilon^2\right), (i = 1, 2, \ldots, g; j = 1, 2, \ldots, e; k = 1, 2, \ldots, r) \tag{17.35}$$

Therefore, we are handling a total of $g \times e$ normal distributions, corresponding to the g genotypes and e environments. The populations may have different means, but they should have the equal variance, which is actually the random error variance across environments. However, unequal error variances may occur if environments are highly heterogeneous. The unequal error variances between environments will be discussed in session 4.5.

Given the phenotypic means of g genotypes and e environments, we can define an overall phenotypic mean $\mu \hat{=} \frac{1}{ge}\sum_{i,j}\mu_{ij}$, phenotypic mean across environments $\overline{\mu}_{i\cdot} \hat{=} \frac{1}{e}\sum_{j}\mu_{ij}$, and environmental mean across genotypes $\overline{\mu}_{\cdot j} = \frac{1}{g}\sum_{i}\mu_{ij}$. We then define the genotypic effect (G_i) as the deviation of each phenotypic mean to the overall mean, environmental effect (E_j) as the deviation of each environmental mean to the overall mean, and genotype by environment interaction (GE_{ij}) as follows:

$$G_i \hat{=} (\overline{\mu}_{i\cdot} - \mu),\ \ E_j \hat{=} \left(\overline{\mu}_{\cdot j} - \mu\right), GE_{ij} \hat{=} \mu_{ij} - \overline{\mu}_{i\cdot} - \overline{\mu}_{\cdot j} + \mu \tag{17.36}$$

Therefore, we can have the following linear model of phenotypic observations:

$$\begin{aligned} &P_{ijk} = \mu_{ij} + \varepsilon_{ijk} = \mu + G_i + E_j + GE_{ij} + \varepsilon_{ijk}, \\ &\varepsilon_{ijk} \sim N\left(0,\ \sigma_\varepsilon^2\right)\ (i = 1, 2, \ldots, g; j = 1, 2, \ldots, e; k = 1, 2, \ldots, r)\ \text{and iid} \end{aligned} \tag{17.37}$$

Variances corresponding to the three kinds of effects indicated in Eq. (17.36) can be defined as well, i.e.,

$$\sigma_G^2 \hat{=} \frac{1}{g-1}\sum_i G_i^2, \sigma_E^2 \hat{=} \frac{1}{e-1}\sum_j E_j^2, \sigma_{GE}^2 \hat{=} \frac{1}{(g-1)(e-1)}\sum_{i,j} GE_{ij}^2 \tag{17.38}$$

17.5.2 Estimation of Effects and Variances

The purpose of multi-environmental trials is to estimate the effects defined in distribution model (17.37) and variances defined in Eq. (17.38), so as to compare the performance of genotypes across environments. Now we will show how the genotypic effect G_i $(i = 1, 2, \ldots, g)$, environmental effect E_j $(j = 1, 2, \ldots, e)$, and interaction effect GE_{ij} $(i = 1, 2, \ldots, g; j = 1, 2, \ldots, e)$ can be estimated from observations P_{ijk} $(i = 1, 2, \ldots, g; j = 1, 2, \ldots, e; k = 1, 2, \ldots, r)$. First, we define the overall sample mean $(\overline{P}\ldots)$, i.e., the mean across the g genotypes, e environments, and r replications. Second, we define sample mean for each genotype and environment $(\overline{P}_{ij\cdot})$, i.e., the mean across the r replications for each genotype and each environment. Third, we define sample mean for each genotype $(\overline{P}_{i\cdot\cdot})$, i.e., the mean across the e environments and r replications for each genotype. Forth, we define sample mean for each environment $(\overline{P}_{\cdot j\cdot})$, i.e., the mean across the g genotypes and r replications for each environment. By calculating the above sample means, the deviation of phenotype to the overall sample mean can be decomposed as the following linear model:

$$P_{ijk} - \overline{P}\ldots = (\overline{P}_{i\cdot\cdot} - \overline{P}\ldots) + (\overline{P}_{\cdot j\cdot} - \overline{P}\ldots) + (\overline{P}_{ij\cdot} - \overline{P}_{i\cdot\cdot} - \overline{P}_{\cdot j\cdot} + \overline{P}\ldots) + (P_{ijk} - \overline{P}_{ij\cdot}) \tag{17.39}$$

The left side of Eq. (17.39) is the deviation of each phenotypic observation to the overall sample mean. On the right side of Eq. (17.39), the first term is the deviation of the genotypic sample mean to the overall mean, which can be used to estimate the genotypic effect defined in Eq. (17.36) or linear model (17.37). The second term is the deviation of the environmental sample mean to the overall mean, which can be used to estimate the environmental effect. The third term quantifies the interaction between genotype and environment, and the last term quantifies the residual random effect. So the effects defined in Eq. (17.36) or linear model (17.37) can be estimated as:

$$\begin{aligned} \hat{\mu} &= \overline{P}\ldots, \hat{G}_i = (\overline{P}_{i\cdot\cdot} - \overline{P}_{\cdot\cdot}), \hat{E}_j = (\overline{P}_{\cdot j\cdot} - \overline{P}\ldots), \\ \hat{GE}_{ik} &= (\overline{P}_{ij\cdot} - \overline{P}_{i\cdot\cdot} - \overline{P}_{\cdot j\cdot} + \overline{P}\ldots) \end{aligned} \tag{17.40}$$

Total sum square (SS_T) corresponds to the deviation on the left side of the model (17.39). Sum square of genotype (SS_G) corresponds to the first term on the right side of the model (17.39). Sum square of environment (SS_E) corresponds to the second term on the right side of the model (17.39). Sum square of interaction (SS_{GE}) corresponds to the third term on the right side of the model (17.39). Sum square of error (SS_ε) corresponds to the forth term on the right side of the model (17.39). That is,

$$\begin{aligned} SS_T &= \sum_{i,j,k} \left(\overline{P}_{ijk} - \overline{P}\ldots\right)^2 = SS_G + SS_E + SS_{GE} + SS_\varepsilon, \\ SS_G &= er\sum_i \left(\overline{P}_{i\cdot\cdot} - \overline{P}\ldots\right)^2, SS_E = gr\sum_j \left(\overline{P}_{\cdot j\cdot} - \overline{P}\ldots\right)^2, \\ SS_{GE} &= r\sum_{i,j} \left(\overline{P}_{ij\cdot} - \overline{P}_{i\cdot\cdot} - \overline{P}_{\cdot j\cdot} + \overline{P}\ldots\right)^2, SS_\varepsilon = \sum_{i,j,k} \left(P_{ijk} - \overline{P}_{ij\cdot}\right)^2 \end{aligned} \tag{17.41}$$

We have a total of $g \times e \times r$ independent observations. Total sum square (SS_T) has a degree of freedom of $ger - 1$. The lost one degree of freedom can be understood as being used in the estimation of the overall sample mean. Sum square of the estimated genotypic effects, i.e., SS_G, has a degree of freedom of $g - 1$, which is equal to the number of independent estimated genotypic effects. Sum square of the estimated environmental effects, i.e., SS_E, has a degree of freedom of $e - 1$, which is equal to the number of independent estimated environmental effects. Sum square of the estimated interaction effects, i.e., SS_{GE}, has a degree of freedom of $(g - 1)(e - 1)$, which is equal to the number of independent estimated interaction effects. There are $g \times e \times r$ estimated residual effects (Eq. 17.39), but they are not completely independent. The degree of freedom of $ge(r - 1)$ can also be understood as the number of independent estimated residual effects. Of course, it can also be found by subtracting $g - 1$, $e - 1$, and $(g - 1)(e - 1)$ from the total degree of freedom $ger - 1$.

Mean square (MS) is defined as the sum square divided by its degree of freedom. That is,

$$\begin{aligned} MS_G &= \frac{SS_G}{g-1}, MS_E = \frac{SS_E}{e-1}, MS_{GE} = \frac{SS_{GE}}{(g-1)(e-1)}, \text{and} \\ MS_\varepsilon &= \frac{SS_\varepsilon}{ge(r-1)} \end{aligned} \tag{17.42}$$

Intuitively, mean square of estimated genotypic effects reflects the magnitude of genotypic variance defined in Eq. (17.38). Mean square of estimated environmental effects reflects the magnitude of environmental variance. Mean square of estimated interaction effects reflects the magnitude of interaction variance. Mean square of the residual effects reflects the magnitude of error variance. In statistics, we can prove

$$\begin{aligned} E(SS_G) &= (g-1)\sigma_\varepsilon^2 + (g-1)er\sigma_G^2, \\ E(SS_E) &= (e-1)\sigma_\varepsilon^2 + g(e-1)r\sigma_E^2, \\ E(SS_{GE}) &= (g-1)(e-1)\sigma_\varepsilon^2 + (g-1)(e-1)r\sigma_{GE}^2, \text{and} \\ E(SS_\varepsilon) &= ge(r-1)\sigma_\varepsilon^2 \end{aligned}$$

Therefore,

$$E(MS_G) = \sigma_\varepsilon^2 + er\sigma_G^2,\ E(MS_E) = \sigma_\varepsilon^2 + gr\sigma_E^2,\ E(MS_{GE}) = \sigma_\varepsilon^2 + r\sigma_{GE}^2,\ \text{and}\ E(MS_\varepsilon) = \sigma_\varepsilon^2 \tag{17.43}$$

From Eq. (17.43), we can see that the expectation of MS_ε is equal to the error variance and therefore is the unbiased estimate of error variance. In addition to genetic variance, error variance is also included in the expectations of MS_G, MS_E, and MS_{GE}, respectively. After some

Table 17.6 ANOVA of multi-environmental phenotyping trials of multiple genotypes

Source of variation	Degree of freedom (DF)	Sum square (SS)	Mean square (MS)	Expected mean square (EMS)
Genotype	$g-1$	SS_G	MS_G	$\sigma_\varepsilon^2 + er\sigma_G^2$
Environment	$e-1$	SS_E	MS_E	$\sigma_\varepsilon^2 + gr\sigma_E^2$
Interaction	$(g-1)(e-1)$	SS_{GE}	MS_{GE}	$\sigma_\varepsilon^2 + r\sigma_{GE}^2$
Error	$ge(r-1)$	SS_ε	MS_ε	σ_ε^2
Total	$ger-1$	SS_T		

algebra manipulations, we can have the following unbiased estimates for the four variances defined in model 17.37 and Eq. (17.38):

$$\hat{\sigma}_G^2 = \frac{1}{er}(\mathrm{MS}_G - \mathrm{MS}_\varepsilon),\ \ \hat{\sigma}_E^2 = \frac{1}{gr}(\mathrm{MS}_E - \mathrm{MS}_\varepsilon),$$
$$\hat{\sigma}_{GE}^2 = \frac{1}{r}(\mathrm{MS}_{GE} - \mathrm{MS}_\varepsilon),\ \text{ and }\ \ \hat{\sigma}_\varepsilon^2 = \mathrm{MS}_\varepsilon \tag{17.44}$$

The above procedure can be summarized in Table 17.6. The following three F-statistics can be calculated to test the significance of the genotypic variation, environmental variation, and interaction variation compared with error variance, respectively:

$$F_G = \frac{\mathrm{MS}_G}{\mathrm{MS}_\varepsilon} \sim F[g-1,\ ge(r-1),]$$
$$F_E = \frac{\mathrm{MS}_E}{\mathrm{MS}_\varepsilon} \sim F[e-1,\ ge(r-1)],\ \text{ and}$$
$$F_{GE} = \frac{\mathrm{MS}_{GE}}{\mathrm{MS}_\varepsilon} \sim F[(g-1)(e-1),\ ge(r-1)]$$

When each replication of the g genotypes is arranged in one relatively homogeneous block in each environment, the block effect in each environment can also be estimated and included in the linear model (17.39). Same as single-environmental trials, the use of block can reduce the random error variance and improve the precision when comparing genotypes. In this case, the deviation of the block mean to the environmental sample mean estimates the block effect (represented by $B_{k(j)}$), i.e.,

$$\hat{B}_{k(j)} = \left(\overline{P}_{\cdot jk} - \overline{P}_{\cdot j\cdot}\right) \quad (j = 1, 2, \ldots, e; k = 1, 2, \ldots, r) \tag{17.45}$$

It should be noted that the block effects have to be defined in each environment. It does not make any sense to talk about the block effects across environments, as field blocks in one environment are totally different from blocks in other environments. Block is not a factor across environment, and block effects are nested in each environment. When the block effects are included, linear model (17.39) becomes

$$P_{ijk} - \overline{P}\ldots = \left(\overline{P}_{\cdot jk} - \overline{P}_{\cdot j\cdot}\right) + \left(\overline{P}_{i\cdot\cdot} - \overline{P}\ldots\right) + \left(\overline{P}_{\cdot j\cdot} - \overline{P}\ldots\right) + \left(\overline{P}_{ij\cdot} - \overline{P}_{i\cdot\cdot} - \overline{P}_{\cdot j\cdot} + \overline{P}\ldots\right)$$
$$+\left(P_{ijk} - \overline{P}_{\cdot jk} + \overline{P}_{\cdot j\cdot} - \overline{P}_{ij\cdot}\right) \tag{17.46}$$

Therefore, ANOVA can be done based on the above model. It can be seen from linear model (17.46) that including the block effect will not affect the estimation of genotypic effects, environmental effects, and interaction effects and will not affect the estimation of genotypic variance, environmental variance, and interaction variance, either. However, it will affect the estimation of residual effects and therefore the error variance. When the block effect is significant, estimated error variance will be lower than that from linear model (17.39). The reduced error variance allows more precise comparison of phenotypic means. In practice, other options are to estimate the block effect using Eq. (17.45), adjust the raw data by the block effect, and apply linear model (17.39) on the adjusted phenotypic observations.

17.5.3 Estimation of Heritability in the Broad Sense

As represented by the linear model (17.37), in multiple environments, phenotype of a quantitative trait for a given genotype or line or family can be decomposed into five parts: (1) overall mean across genotypes, environments, and replications; (2) genotypic effect of the specific genotype; (3) environmental effect of the specific environment; (4) genotype by environment interaction effect; and (5) random residual error. That is,

$$P = \mu + G + E + GE + \varepsilon \tag{17.47}$$

where overall mean, genotypic effects, environmental effects, and interaction effects are assumed to be unknown parameters (or fixed effects), and residual error is assumed to be a random variable. When random error has a normal distribution, the phenotypic variance ${\sigma_P}^2$ is equal to the sum of genotypic variance ${\sigma_G}^2$, environmental variance ${\sigma_E}^2$, interaction variance ${\sigma_{GE}}^2$, and error variance ${\sigma_\varepsilon}^2$. In session 4.2, we have seen that ANOVA can give the unbiased estimates of those variances. Therefore, we can have the unbiased estimate of phenotypic variance as follows:

$$\sigma_P^2 = \sigma_G^2 + \sigma_E^2 + \sigma_{GE}^2 + \sigma_\varepsilon^2$$

In genetics, we are more concerned about the genetic variance and genotype by environment interaction. So environmental variance is normally excluded from phenotypic variance, i.e.,

$$\sigma_P^2 = \sigma_G^2 + \sigma_{GE}^2 + \sigma_\varepsilon^2 \tag{17.48}$$

Similar to single-environmental trials, proportion of genetic variance over phenotypic variance is defined as the heritability in the broad sense, represented by H^2, i.e.,

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_{GE}^2 + \sigma_\varepsilon^2} \tag{17.49}$$

Therefore, in applying the estimates of genotypic, interaction, and error variances in Eq. (17.49), we can estimate the heritability in the broad sense of quantitative traits.

The heritability estimated by the formula (17.49) is based on single observations. When genetic analysis is based on phenotypic mean across environments and replications, i.e., $\overline{P}_{i\cdot\cdot}$, Eqs. (17.47), (17.48), and (17.49) become

$$\overline{P} = \mu + G + \overline{\varepsilon} \tag{17.50}$$

$$\sigma_{\overline{P}}^2 = \sigma_G^2 + \frac{1}{er}\sigma_\varepsilon^2 \tag{17.51}$$

$$H^2 = \frac{\sigma_G^2}{\sigma_{\overline{P}}^2} = \frac{\sigma_G^2}{\sigma_G^2 + \frac{1}{er}\sigma_\varepsilon^2} \tag{17.52}$$

17.5.4 An Example on Yield of Ten Maize Inbred Lines in Three Environments

Ten maize recombination inbred lines (RILs) were evaluated across three environments, and the randomized block design (RBD) was used in each environment. In each environment, three replications of the ten lines are arranged in three homogenous field blocks. Yield was measured on each plot of replication, and raw data is given in Table 17.7. Please be noted that Table 17.4 actually shows the results from environment I. Let's first ignore the issue of heterogeneous environments and assume that the three environments have equal error variance. The issue of heterogeneous environments will be discussed in session 8.5.

Row mean represents the phenotypic mean of each RIL, which is shown in the second last column in Table 17.7. Column mean across the three replications represents each environmental mean, which is shown in the fourth last row in Table 17.7. Mean across the ten RILs, three environments, and three replications is the overall mean, which is equal to 3.13 (Table 17.7). The deviation of phenotypic mean to the overall mean is the genotypic effect, which is shown in the last column in Table 17.7. The deviation of environmental mean to the overall mean is the environmental effect, which is shown in the last third row in Table 17.7. Obviously, the ten genotypic effects have a sum of 0 and so have the three environmental effects. The interaction effects can be calculated by the formula (17.40) (not shown). Interaction effects have a total number

Table 17.7 Yield performance of ten maize inbred lines in three replications and three environments

	Environment I			Environment II			Environment III				
Genotype	Rep I	Rep II	Rep III	Rep I	Rep II	Rep III	Rep I	Rep II	Rep III	Phenotypic mean $(\overline{P}_{i\cdot})$	Genotypic effect $(\hat{G}_i)$
RIL1	2.56	2.66	2.43	2.25	2.34	2.25	4.09	4.19	4.01	2.976	−0.151
RIL2	2.66	2.50	2.75	2.61	2.21	2.75	1.35	2.12	2.81	2.418	−0.709
RIL3	2.93	2.97	2.70	2.79	3.11	3.06	3.89	3.20	3.15	3.089	−0.038
RIL4	2.57	2.21	1.80	4.01	3.69	3.23	3.83	4.73	4.33	3.378	0.251
RIL5	3.06	2.61	2.72	3.60	3.11	3.15	3.66	3.71	3.89	3.279	0.152
RIL6	1.94	2.16	2.14	2.16	2.93	1.71	4.27	3.17	4.32	2.756	−0.371
RIL7	1.85	1.69	2.25	2.39	2.03	2.57	3.73	3.65	1.88	2.449	−0.678
RIL8	3.83	3.58	3.80	3.74	5.04	4.46	3.33	3.30	3.38	3.829	0.702
RIL9	4.32	4.12	4.14	4.91	4.32	3.96	4.06	3.90	4.29	4.224	1.097
RIL10	2.81	3.33	2.81	3.69	3.15	3.51	2.89	1.55	2.12	2.873	−0.254
Environmental mean $(\overline{P}_{\cdot j\cdot})$	2.797			3.158			3.427			$\overline{P}\ldots = 3.127$	
Environmental effect $(\hat{E}_j)$	−0.330			0.031			0.300				
Block mean $(\overline{P}_{\cdot jk})$	2.853	2.783	2.754	3.215	3.193	3.065	3.510	3.352	3.418		
Block effect $(\hat{B}_{k(j)})$	0.056	−0.014	−0.043	0.057	0.035	−0.093	0.083	−0.075	−0.009		

of $g \times e$, with a sum of 0. Additionally, the g effects for each environment have a sum of 0, the e effects for each genotype have a sum of 0, and therefore the number of independent effects is equal to the degree of freedom of $(g-1) \times (e-1)$.

Column mean across the ten genotypes represents the block mean, which is shown in the second last row in Table 17.7. The deviation of block mean to the environmental sample mean is the block effect, which is shown in the last row in Table 17.7. So we have block effects for the three replications and the three environments. Obviously, the three block effects in each environment have a sum of 0.

When block effects are also ignored, Table 17.8 shows the combined ANOVA across the three environments. Actually, SS of genotype is equal to $e \times r$ times of the sum of the squared genotypic effects. SS of environment is equal to $g \times r$ times of the sum of the squared environmental effects. SS of interaction is equal to r times of the sum of the squared interaction effects. It can be seen from Table 17.8 that the ten RILs show significant difference on the yield. In addition, environmental effects and interaction effects are highly significant as well. From the four mean squares, error variance is estimated at 0.165, environmental variance at 0.094, genotypic variance at 0.312, and interaction variance at 0.220. From estimated variances, heritability in the plot level is estimated at 44.8 %, and heritability in the phenotypic mean level is estimated at 94.5 %.

17.5.5 Estimation of Genotypic Value in Heterogeneous Environments

In multiple-environmental trials, it is generally assumed that different genotypes in a specific environment have the same error variance. This assumption may be unrealistic when environmental conditions are heterogeneous or when the data span a long time period. Several sources of heterogeneous variances are identified to make the environments heterogeneous, including temperature, water, soil, pest, etc. There are several useful tests of the homogeneity of variance assumption. Here we show how to use Bartlett's test to check if variances are homogenized. Let $\hat{\sigma}^2_{\varepsilon_j}$ and df_{ε_j} be error variance for the jth environment and its degree of freedom, respectively, and then null hypothesis and alternative hypothesis are

$$H_0 : \sigma^2_{\varepsilon_1} = \sigma^2_{\varepsilon_2} = \cdots = \sigma^2_{\varepsilon_e}, \text{ and}$$
$$H_A : \text{at least two of } \sigma^2_{\varepsilon_1}, \sigma^2_{\varepsilon_2}, \ldots \text{ and } \sigma^2_{\varepsilon_e} \text{ are not equal.}$$

Under null hypothesis, the combined error variance ${\sigma_\varepsilon}^2$ can be obtained by individual error variances of the e environments, that is,

$$\sigma^2_\varepsilon = \frac{1}{\sum_j df_{\varepsilon_j}} \sum_j df_{\varepsilon_j} \times \sigma^2_{\varepsilon_i} \tag{17.53}$$

Bartlett's statistics approximately follows χ^2 distribution with degree of freedom $e-1$, that is,

$$\chi^2 = \left(\sum_j df_{\varepsilon_j}\right) \ln\left(\sigma^2_\varepsilon\right) - \sum_j df_{\varepsilon_j} \times \ln\left(\sigma^2_{\varepsilon_i}\right) \sim \chi^2(e-1) \tag{17.54}$$

Under heterogeneous environments, the mean performance of one genotype is assumed to be μ, error variance in the jth environment is $\sigma^2_{\varepsilon_j}$, and P_j is its phenotypic value in the jth environment. Therefore, the linear model is

Table 17.8 ANOVA of the multi-environmental trial shown in Table 17.7

Source	DF	SS	MS	Variance	*F* value	*P* value
Environment	2	5.996	2.998	0.094	18.219	0.000
Genotype	9	26.753	2.973	0.312	18.064	0.000
Interaction	18	21.686	1.205	0.220	7.322	0.000
Error	60	9.873	0.165	0.165		
Total	89	64.308				
R^2 (%)	84.647					
LSD ($P = 0.05$)	1.087					
LSD ($P = 0.01$)	1.489					
H^2 per plot	0.448					
H^2 per mean	0.945					

$P_i = \mu + \varepsilon_j, \varepsilon_j \sim N\left(0,\ \sigma^2_{\varepsilon_j}\right) (j = 1, 2, \ldots, e)$ and independent

In this case, the simple mean $\overline{P}. = \frac{1}{e}\sum_j P_j$ is still an unbiased estimation of μ but not the best one. That is to say, there are other estimates having smaller variance than the simple mean. By calculating the variance of the linear combination of P_j ($j = 1, 2, \ldots, e$), BLUE of μ can be found as

$$\hat{\mu} = \sum_j w_j P_j, \quad \text{where}$$

$$w_j = crdisplaystyle \frac{\frac{1}{\sigma^2_{\varepsilon_j}}}{\frac{1}{\sigma^2_{\varepsilon_1}} + \frac{1}{\sigma^2_{\varepsilon_2}} + \cdots + \frac{1}{\sigma^2_{\varepsilon_e}}} \tag{17.55}$$

The variance of $\hat{\mu} = \sum_j w_j P_j$ can be found as

$$V(\hat{\mu}) = \frac{1}{\frac{1}{\sigma^2_{\varepsilon_1}} + \frac{1}{\sigma^2_{\varepsilon_2}} + \cdots + \frac{1}{\sigma^2_{\varepsilon_e}}}. \tag{17.56}$$

The variance given in Eq. (17.56) is the least among all possible unbiased linear combinations of P_j ($j = 1, 2, \ldots, e$). If and only if environments are homogeneous, $V(\hat{\mu})$ and $V\left(\overline{P}.\right)$ are equal. That is to say, when environments are heterogeneous, weighted mean is a better estimate than simple mean, in the sense of least variance. When an environment has smaller error variance, a higher weight should be given for this environment.

To illustrate the effectiveness of weighted mean, we assume that there are two environments and $\sigma^2_{\varepsilon_2} = s\sigma^2_{\varepsilon_1}$. Then, we have

$$V(\hat{\mu}) = \frac{s}{1+s}\sigma^2_{\varepsilon_1},\ V\left(\overline{P}\right) = \frac{1}{4}(1+s)\sigma^2_{\varepsilon_1},\ \text{and}\ \frac{V(\hat{\mu})}{V\left(\overline{P}\right)} = \frac{4s}{(1+s)^2}$$

We can clearly see the ratio of variance of weighted mean and simple mean in Fig. 17.3. When $s = 1$, that is, $\sigma^2_{\varepsilon_2} = \sigma^2_{\varepsilon_1}$, $V(\hat{\mu}) = V\left(\overline{P}\right)$. When $s \neq 1$, $V(\hat{\mu})$ is always smaller than $V\left(\overline{P}\right)$.

$\sigma^2_{\varepsilon_1} \neq 0$ and $\sigma^2_{\varepsilon_2} = 0$ ($s = 0$) are an extreme case, which indicates that the second environment does not have any error. In this case, the observation in the second environment is equal to the phenotypic mean. The error variance in the first environment is nonzero, which indicates that the observation in the first environment may be deviated from the phenotypic mean. In this case, the observation in the second environment is the best estimate of μ. Including observations from the first environment may cause deviation from the phenotypic mean.

$\sigma^2_{\varepsilon_1} \neq 0$ and $\sigma^2_{\varepsilon_2} = \infty$ represent another extreme case. Observations in the second environment have nothing to do with μ. In this case, observation in the second environment is complete random error, which does not contain any useful information about the phenotypic mean to be estimated. Including observations from the second environment may cause more deviation from the phenotypic mean. Thus observations in the first environment are the best estimate of μ.

Now, we revisit the data in Table 17.7. Table 17.9 summarizes the ANOVA results in the three environments. The three error variances were estimated respectively at 0.0473, 0.1525,

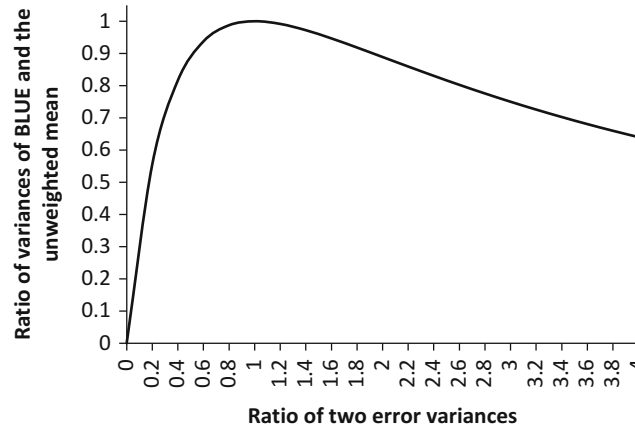


Fig. 17.3 The variance ratio of BLUE and the simple unweighted mean

Table 17.9 ANOVA in each environment using data in Table 17.7

	Mean square				Estimated variance		Heritability	
Environment	Genotype	Error	*F* value	DF of error	Genotype	Error	Per plot	Per mean
I	1.530	0.047	32.346	20	0.494	0.047	0.913	0.969
II	2.044	0.153	13.404	20	0.630	0.153	0.805	0.925
III	1.809	0.294	6.154	20	0.505	0.294	0.632	0.838

and 0.2939, with the same degree of freedom of 20. If the null hypothesis $H_0 : \sigma^2_{\varepsilon_1} = \sigma^2_{\varepsilon_2} = \sigma^2_{\varepsilon_3}$ is true, the combined error variance is estimated $\hat{\sigma}^2_{\varepsilon} = 0.1646$ by Eq. (17.53). The Bartlett's χ^2 statistic defined in Eq. (17.54) has a value of 14.86, and its degree of freedom is 2. Hence the significance probability can be found at $P = 0.0003$, which is highly significant. The high significance from the χ^2 test indicates the heterogeneity among the three environments. In theory, it is not appropriate to conduct the combined ANOVA as Table 17.8, when the environments are heterogeneous. Instead, ANOVA should be conducted for each environment, as shown in Table 17.9.

For comparison, Table 17.10 gives simple means and BLUE (or weighted means) of the ten RILs and ranks of the ten RILs from simple mean and BLUE. Weights in BLUE are 0.68, 0.21, and 0.11 for the three environments in calculating BLUE (last row in Table 17.10). Environment I has the least error variance of 0.047 (Table 17.9) and therefore has the highest weight in BLUE. Environment III has the largest error variance of 0.294 (Table 17.9) and therefore has the lowest weight in BLUE. The use of weighted mean does not change the ranks of the two top RILs but gives quite different ranks for other RILs. As indicated before, BLUE has the least variance compared to any other unbiased linear estimates. Considering the highly heterogeneous environments, BLUE given in Table 17.10 is expected to be much closer to the true phenotypic mean of each RIL and therefore should be used in further genetic studies, such as QTL mapping.

The sample mean across the three replications of each RIL has a variance at 0.016 in environment I, 0.051 in environment II, and 0.098 in environment III (Table 17.10), which is equal to the estimated error variance divided by the number of replications. Therefore, variance of the simple mean and variance of BLUE and their standard error (SE) are

Table 17.10 Comparison of simple mean and BLUE and using data in Table 17.7

	Environment						
Genotype	I	II	III	Simple mean	Rank	BLUE	Rank
RIL1	2.55	2.28	4.10	2.976	6	2.662	7
RIL2	2.64	2.52	2.09	2.418	10	2.555	8
RIL3	2.87	2.99	3.41	3.089	5	2.954	5
RIL4	2.19	3.64	4.30	3.378	3	2.726	6
RIL5	2.80	3.29	3.75	3.279	4	3.007	3
RIL6	2.08	2.27	3.92	2.756	8	2.321	9
RIL7	1.93	2.33	3.09	2.449	9	2.141	10
RIL8	3.74	4.41	3.34	3.829	2	3.838	2
RIL9	4.19	4.40	4.08	4.224	1	4.222	1
RIL10	2.98	3.45	2.19	2.873	7	2.993	4
Variance	0.016	0.051	0.098				
Weight	0.680	0.211	0.109				

$$V(\hat{\mu}) = \frac{1}{\frac{1}{0.016} + \frac{1}{0.051} + \frac{1}{0.098}} = 0.011,$$

$$\mathrm{SE}(\hat{\mu}) = \sqrt{0.011} = 0.104, V(\overline{P})$$

$$= \frac{1}{3} \times 0.016 + \frac{1}{3} \times 0.051 + \frac{1}{3} \times 0.098$$

$$= 0.055, \quad \mathrm{SE}(\overline{P}) = \sqrt{0.055} = 0.234$$

Obviously, the weighted mean has much smaller variance and SE compared with the unweighted mean. The error variance estimated above can be used in significance test between the ten RILs, as normally done in ANOVA.

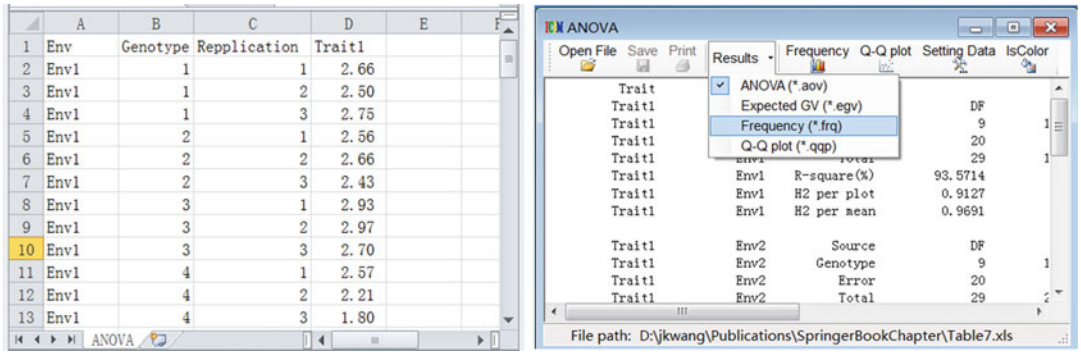
17.6 A Computer Tool for Analyzing Multi-environmental Trials

QTL IciMapping (freely available from www.isbreeding.net) was an integrated software for linkage map construction and QTL mapping. For multi-environmental trials, a tool called ANOVA is implemented in the software to estimate the genetic variance and heritability in broad sense from phenotypic data. The data format can be in CSV, XLS, or XLSX. If in format of XLS or XLSX, the sheet name must be "ANOVA" (left of Fig. 17.4). In sheet ANOVA, the first row is for Environment, the second row is for Genotype, the third column is for Replication, the forth column is for the first trait, the fifth column is for the second trait, and so on (left of Fig. 17.4). The first three columns can be either number or string. Columns for traits must be numbers. Missing trait values were denoted as −100.00.

From output, the users can find standard ANOVA tables for each environment and combined analysis across environments for each trait in AOV file, and the expected genotypic values for each environment and combined analysis for each trait in EGV file. Besides, frequency histogram (FRQ file) and Q-Q plot (QQP file) can be shown by selecting corresponding menus for raw phenotype and expected genotype per replication, per trait, and per environment (Right of Fig. 17.4).

17.6.1 Objectives in Phenotyping Complex Traits

Genotypic value can be estimated by marker loci or by known quantitative trait loci. But even if marker loci or quantitative trait loci are not analyzed, the relative magnitudes of additive, dominance, and epistatic effects across unknown loci can be estimated from an analysis of the phenotypic value. In classic quantitative genetics, the number of genes controlling the trait of interest can be estimated by Castle-Wright formula (Sect. 17.2.3). The segregation analysis can



	A	B	C	D	E
1	Env	Genotype	Repplication	Trait1	
2	Env1	1	1	2.66	
3	Env1	1	2	2.50	
4	Env1	1	3	2.75	
5	Env1	2	1	2.56	
6	Env1	2	2	2.66	
7	Env1	2	3	2.43	
8	Env1	3	1	2.93	
9	Env1	3	2	2.97	
10	Env1	3	3	2.70	
11	Env1	4	1	2.57	
12	Env1	4	2	2.21	
13	Env1	4	3	1.80	

Trait			
Trait1			DF
Trait1			9
Trait1			20
Trait1			29
Trait1	Env1	R-square(%)	93.5714
Trait1	Env1	H2 per plot	0.9127
Trait1	Env1	H2 per mean	0.9691
Trait1	Env2	Source	DF
Trait1	Env2	Genotype	9
Trait1	Env2	Error	20
Trait1	Env2	Total	29

Fig. 17.4 Multi-environmental phenotyping data in Excel (*left*) and the interface of the ANOVA computing tool (*right*)

be used to estimate genetic parameters of the variation of a quantitative trait, including additive and dominance effects, additive and dominance variance, and heritability for both major genes and polygenes (Gai and Wang 1998). Therefore, only by phenotypic data, we can distinguish the effects of major genes from polygenes and/or environments, which is important for understanding the expression of a major gene in relation to its genetic background and for predicting the segregation of a cross in breeding.

17.6.2 The Three Basic Principles of Field Experimental Design in Phenotyping Complex Traits

In field experiments, environmental condition may vary from one stage of the experiment to the next. Some factors may not be possible to isolate from others, thus forcing the investigation of several factors jointly. On top of that, measurement errors may introduce unwanted error into the system. Therefore, precautionary measures need to be taken. To design the experiment in a better way, R. A. Fisher has enumerated three principles of experimental designs (Fisher 1926): (1) the principle of local control, (2) the principle of randomization, and (3) the principle of replication. These are discussed in details in Chap. 16 and briefly described below.

The principle of local control eliminates the variability caused by extraneous factors can be measured. This means that we should plan the experiment in a manner that we can perform a three-way ANOVA, in which the total variability of the data is divided into four components attributed to treatments (genotype in our case), environments (in our case), extraneous factor (e.g., soil fertility), and experimental error. In other words, in each environment, we first divide the field into several homogeneous parts, known as blocks, and then each such block is divided into parts equal to the number of genotypes. In general, blocks are the levels at which we hold an extraneous factor fixed, so that we can measure its contribution to the total variability of the data by means of a three-way ANOVA (Eq. 17.46 in Sect. 17.4.2). For increasing the statistical accuracy of the experiment, the principle of replication is required in which the experiment is repeated more than once. Thus, each treatment is applied in many experimental units instead of one. For example, when considering multiple genotypes in one environment (Sect. 17.3), more replications give more precise estimate of the true genotype (Eq. 17.25). Sometimes the entire experiment can be repeated several times for better results. It should be remembered that replication is introduced in order to increase the precision of a study, that is to say, to increase the accuracy with which the main effects and interactions can be estimated. Finally, principle of randomization provides protection against the effects of extraneous factors by randomization, and each treatment has equal opportunity to get a

place where soil fertility is good or bad. For instance, if we grow one variety of maize, say, in the first half of the parts of a field and the other variety is grown in the other half, then it is just possible that the soil fertility may be different in the first half in comparison to the other half. If this is so, our results would not be realistic. In such a situation, we may assign the variety of rice to be grown in different parts of the field on the basis of some random sampling technique. Through the application of the principle of randomization, we can have a better estimate of the well-known experimental error.

17.6.3 Quality Control of Phenotype

Phenotypic values are usually measured in multiple environments/locations/years, each with several replications. Measurement procedure is fraught with sources of potential error, which may arise in the observer, in the plant, or in the overall application of the technique. Therefore quality assurance and quality control of phenotype are essentially required to insure the success of genetic study. Generally, quality control of phenotype should be conducted right after the phenotypic values are out of the measurements. Maximum value, minimum value, mean, variance, histogram, variance of replications, heritability in broad sense, etc. are the easiest and most effective statistics to evaluate the raw phenotypes.

- Outliers (or unusual values) are values that lie outside the usual range of phenotype of the trait of interest. They can seriously affect the results of analyses. There are two aspects in dealing with outliers, identifying them and dealing with them. There are formal tests for detecting outliers (Miller 1993; Sokal and Rohlf 1995), but they can be easily highlighted by the distribution of phenotype, that is, histogram plot. Outliers would make either maximum value or minimum value to be anomalous and make variance abnormally large. Once we identify outliers, we should first check to make sure they are not a mistake, such as an error typing in your data or in writing values down. They often show up as impossible values, for example, −5 cm for plant height. If you can classify an outlier as a mistake, it should be deleted. If you have no reason to suspect an outlier as being a mistake, you can do the genetic study without the outlier to see how much they influence the outcome of the analysis. If the conclusions are altered, then you should try and determine why those values are so different. Perhaps there was contamination during pollen; plants were from different subpopulation, etc.
- The shape of the distribution of phenotype can be examined by plotting a histogram. Is the distribution symmetrical or skewed? Is it unimodal or multimodal? We may find a priori like biological or physiological reasons to explain this distribution. In some cases, genetic models for the trait of interest can be estimated by the distributions (Gai and Wang 1998; Wang et al. 2001).
- The large variance of replications should be argued. Replication demonstrates the results to be reproducible, at least under the current experimental conditions. Large variance of replications indicated that the repetitiveness of the experiments is poor, the precision for estimates of genotype mean is low, and the experimental error variance is large, which may cause the low heritability in broad sense.
- Heritability in broad sense is low. Heritability is a concept that summarizes how much of the variation in a trait is due to variation in genetic factors. We have demonstrated how to estimate heritability in broad sense by phenotypic values in previous sections. A low heritability means that of all observed variations, a small proportion is caused by variation in genotypes (Visscher et al. 2008). That is to say, the heritability of a group of individuals with relatively similar heredities is relatively low, and the phenotype of an individual is not a good predictor of the genotype. In many gene-mapping experiments, the probability of detecting a gene of large effect increases with heritability (Bradford and Famula 1984; Oliver et al. 2005; Weedon et al. 2007). Therefore, low heritability implies that the follow-up genetic study may be not efficient.

17.6.4 Fixed Effect or Random Effect

Analysis based on phenotypic values in Sects. 17.2, 17.3, 17.4, and 17.5 is under assumption that all interested effects, including genotypic effect, environmental effect, genotypic by environment interaction effect, and block effect, are fixed effects. It is natural to ask when and which explanatory variables (also called independent variables) to give random effects. Conceptually, effects of variables might be treated as random if we can think of the levels of the variable that we included in the study as a sample drawn from some larger population of levels that could (in principle) have been selected. Practically, one key difference between fixed and random effects is in the kind of information we want from the analysis of the effects.

In the case of fixed effects, we are usually interested in making explicit comparisons of one level against another. For example, we would want to compare the yield mean in Beijing to that in New York in an experiment. If explicit comparison of the levels of a variable against one another is the goal of the research, then the levels of the variable are usually treated as "fixed." If, on the other hand, our primary interest is in the effects of other variables or treatments across the levels of a factor (e.g., the effect of block on yield, across genotypes from three environments), that is to say, we assumed that the block effect varies randomly within the population of environments, and the researcher is interested to test and estimate the variance of these random effects across this population. Then the block variable might be treated as a "random" effect. In this chapter, we assumed that all the related effects are fixed.

17.6.5 Conclusion

Phenotypic variability may be caused by genotype and environmental factors. Therefore plant geneticists are interested to dissect phenotype and to identify the genes that play important roles in the inheritance of phenotype. They try to explain the role of those genes in relation to one another and in relation to the environment. Genetic mapping correlates the phenotype with the genotype of genetic markers, which are expected to be located close to the genes (or genomic regions) of interest. The relationships between the phenotype and gene of interest can be weakened if phenotypic variability is underestimated or overestimated. Therefore, traits should be measured reproducibly on a large number of samples, and biometrical techniques can be useful to determine the true genotypic value. This can help to locate, enumerate, and annotate genes and to assign known or putative biochemical functions. However, only about two-thirds of all genes have an assigned biochemical function, and only a fraction of those are associated with a phenotype. Therefore some efforts are needed on phenomics (Bochner 2003) in order to accelerate the pace of the discovery of genes using advanced tools and techniques of biometrics.

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Harnessing Genomics Through Phenomics

18

Reyazul Rouf Mir, Neeraj Choudhary, Bikram Singh, Irshad Ahmad Khandy, Vanya Bawa, Parvez Sofi, Aijaz Wani, Sumita Kumari, Shalu Jain, and Ajay Kumar

Abstract

Plant genetics and genomics have revolutionized agricultural research, and a vast amount of genomics resources have been developed in crop plants. However, these genomics resources could not be utilized with their full potential in genetic improvement of crop plants especially for the improvement of complex quantitative traits related to biotic and abiotic stresses and the outcome is still far from satisfactory. Among several reasons, the lack of availability of precise and high-throughput phenotyping tools are cited as the major one, as poor phenotyping has led to poor results in gene/QTL discovery for genomics-assisted breeding applications. During the recent past, high-throughput precise phenotyping tools and techniques have been developed, which led to development of a number of phenomics platforms. These phenomics platforms can help us to collect high-quality accurate phenotyping data necessary for harnessing the potentiality of genomics resources through genetic dissection of complex quantitative traits including discovery of new gene/QTL, identification of gene function, and genomics selection. This chapter focuses on recent developments in the area of phenomics and provides an overview on the practical use of genomics through crop phenomics.

Keywords

Phenomics • High-throughput and precise phenotyping • Genomics • QTL/gene discovery • Genomics-assisted breeding

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18.1 Introduction

Plant genetics and genomics have revolutionized agricultural research. The genomics revolution started after the sequencing of model weed plant *Arabidopsis thaliana* in 2000. Tremendous progress has been made in the area of genomics since then. These advances include development and use of molecular marker technologies, development of molecular genetic and physical maps, gene discovery through various approaches, cloning of genes for agriculturally important traits, and sequencing tools and techniques like next-generation sequencing (NGS) (Mir and Varshney 2013; Mir et al. 2013; Gupta et al. 2013b). Use of advanced genomics tools and technologies has enhanced the precision of conventional breeding programs leading to the development of superior cultivars with enhanced resistance/tolerance to biotic and abiotic stresses (Varshney et al. 2012; Mir et al. 2012). However, the cause of concern for the scientific community is the declining growth/stagnation in production of major food crops in the last few decades. This stagnation poses a huge challenge to increase 50–70 % production by 2050 (Furbank and Tester 2011) to feed the extra billion population. The most fertile agricultural land is facing competition from the industrialization, and the present area under cultivation faces various biotic/abiotic stresses, viz., fungal and bacterial diseases, heat, salinity, and drought stresses. All these biotic and abiotic stresses exert tremendous survival pressure on crop germplasm (Mir et al. 2012). Under the prevailing conditions and available resources, new plant varieties with desired traits and high yield potential need to be developed. This can be achieved through better understanding of the genetic makeup of plants and their phenotype and correlation between the two along with environmental challenges. It is important to mention here that despite the achievement of genomics revolution during the past few decades, the outcome is still far from satisfactory as the existing technologies are not effective in enhancing crop productivity. Among several reasons, one of the possible reasons could be the lack of availability of precise and high-throughput phenotyping tools, which has led to poor results in gene/QTL discovery for genomics-assisted breeding applications. The genomics application in agriculture can be made more effective and meaningful by laying stress on the importance of crop phenotyping/phenomics (Pieruschka and Poorter 2012). This chapter focuses on how phenomics can be integrated to harness the potential of vast amount of developed genomic resources in crop plant species.

18.2 Plant Phenotyping

Plant phenotyping considered an age-old practice is the evaluation of agronomic characters of various cultivars to access the genotype. However, phenotyping is considered a more tedious/time-consuming job than genotyping owing to the variations caused by environment interactions and other changes (Pieruschka and Poorter 2012). To get more precise and authentic knowledge of the phenotype of plant traits, a better version of phenotyping is introduced called "phenome," counterpart of "genome." If genome is a total sum of genes present in an individual, then phenome is all the expressed traits of the individual (Pieruschka and Poorter 2012). Sometimes, plant phenomics is considered as "high-throughput plant physiology" or "plant physiology with new cloths" (Furbank and Tester 2011) which helps on the extensive study of complete life cycle with least destruction. The era of phenomics is believed to help us in unlocking the information coded in plant genomes (Finkel 2009). Phenomics initiatives have been undertaken in several parts of the world including Germany, France, the United Kingdom, Australia, The Netherlands, Hungary, and Israel. Some progress has been already made in the area of phenomics, but much needs to be done to achieve fruitful results in using genomics and molecular breeding approaches like QTL interval mapping, association mapping, genome-wide association studies (GWAS), QTL cloning, marker-assisted selection (MAS), marker-assisted recurrent selection (MARS), and genomic selection (GS) or genome-wide selection (GWS) for crop improvement programs (Cobb et al. 2013).

18.3 Genomics for Crop Improvement

Conventional methods of plant breeding have made a significant contribution to crop improvement, but the pace of progress has slowed down drastically in the recent past as these methods were less effective in targeting complex traits like grain yield, quality traits, and abiotic stress tolerance such as drought, salinity, heat, etc. Conventional methods were unable to precisely locate the position of genes controlling the phenotypic expression and their individual main/epistatic effect. Genomics brings new and powerful tools to solve difficult problems until unresolved for a major yield breakthrough. Major advances in genomics tools and technologies have made gene discovery possible in crop plants and help in better understanding/genetic dissection of gene networks/complex quantitative traits that contribute to the development of a superior genotype. Rapid development of the functional genomics and gene technologies that occur over the past decade has led to the functional analysis of the genomes of major crops to enter into the high-throughput stage. Dozens of key genes of major crop species (rice, wheat, etc.) have been cloned and characterized for their function in controlling important agronomic traits. Molecular markers have enabled us to prepare dense molecular maps in major crops and mapping of QTLs/genes. Cheap and high-throughput DArT, SNP, SFP, and GBS markers made GWS and GWAS increasingly affordable and applicable on a broader scale. Low sequencing cost due to emergence of what we call NGS technologies has provided new opportunities for gene discovery and allele mining (Varshney et al. 2009). Although the integration of molecular approaches in breeding programs of several crops still faces a number of limitations (e.g., lack of SNP platforms, high cost of high-throughput phenotyping platforms, poor understanding of gene functions and interactions, etc.), the role of genomics-assisted crop improvement (GACI) will become increasingly important to achieve the selection gains that will be required to adequately meet the growing needs of mankind in the next decades. Thus, the use of molecular and genomics tools in plant breeding makes it possible to design the model plant of the crop species having high yield, better quality, and high resistance/tolerance to biotic and abiotic stresses. This makes the concept called breeding by design possible that aims to control all allelic variations for all genes of agronomic importance. This concept can be realized through a combination of various approaches like genetic mapping, high-resolution chromosome haplotyping, and extensive phenotyping (Peleman and Voort 2003). One of the challenges of genomics is its translation into crop improvement after the discovery/validation of QTLs/genes. The QTLs/genes need to be introgressed into the most popular varieties of the crop species through modern marker-assisted breeding (MAB) approaches, sometimes called as GACI (Varshney and Tuberosa 2007). Recently, a new molecular breeding approach called GWS has been found useful for breeding yield and other complex traits (Bernardo 2010). The main advantage of GWS is that it does not require any a priori information or knowledge about the marker-trait associations as is required for MAS, marker-assisted backcrossing (MABC), and MARS. In addition, GWS considers the effects of all markers spread all over the genome thus capturing most of the additive variation underlying complex quantitative traits. GACI offers unprecedented opportunities to identify major loci influencing the targeted traits and to select for plants with the desirable combination of alleles via MAS, MABC, or MARS (Mir et al. 2012). The QTL mapping techniques have been used for the discovery and identification of QTLs/genes for a number of agriculturally important traits including yield, quality traits, and biotic and abiotic stress resistance/tolerance in almost all the major crop species (Gupta et al. 2013a). Therefore, genomics tools and technological advances have enhanced the precision in conventional breeding programs and in several cases led to the development of superior cultivars with enhanced resistance/tolerance to biotic/abiotic stresses through genomics-assisted breeding (Varshney et al. 2012).

However, despite these advances in genomics tools and techniques, the information that became available has not been adequately exploited due to outdated and laborious phenotyping tools. It became apparent that the high-throughput phenotyping is an important new bottleneck in crop breeding and plant biology.

18.4 High-Throughput Phenotyping

In the era of phenomics, high-throughput precise phenotyping helps us to collect high-quality accurate phenotyping data. The high-quality phenotypic data is very important and useful for meaningful genetic dissection and genomics-assisted breeding applications including (i) QTL interval mapping, (ii) association mapping, (iii) GWAS, (iv) QTL cloning, (v) QTL meta-analysis, (vi) MAS, (vii) MARS, and (viii) GS or GWS (Welcker et al. 2011; Tuberosa 2012; Cobb et al. 2013; Fig. 18.1). Recently, an International Plant Phenomics Initiative was launched to address crop productivity (http://www.plantphenomics.org/). The earlier use of invasive or destructive methods of plant phenotyping is now giving way to high-throughput precise non-destructive imaging techniques. Several phenomics platforms are now available around the world with good facilities allowing scientists new windows into the inner workings of living

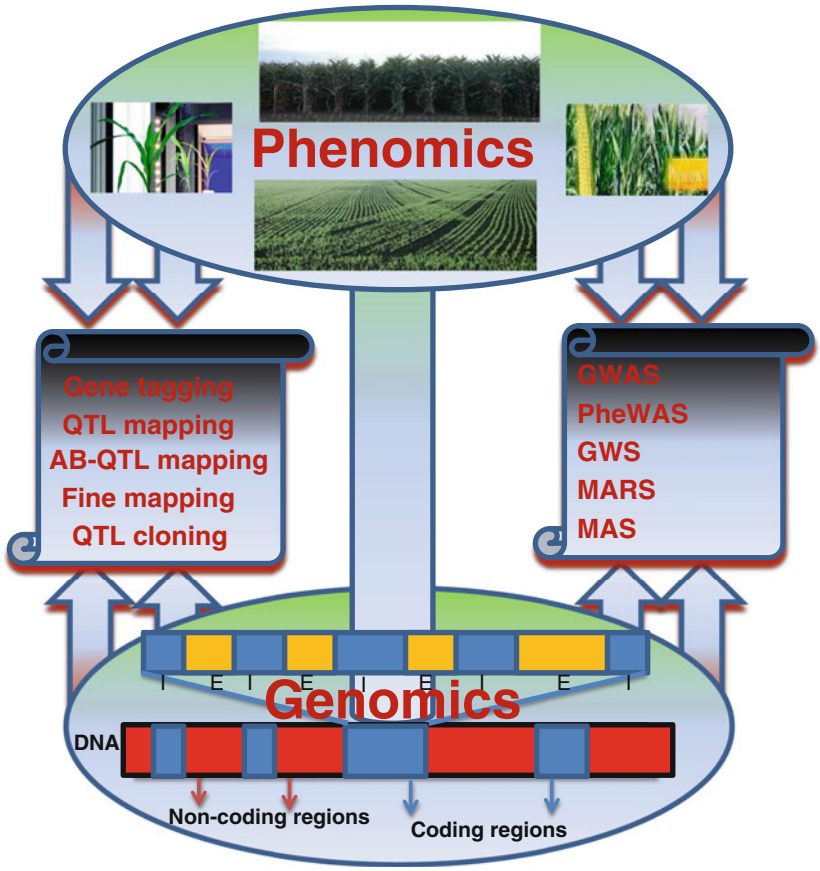

Fig. 18.1 Diagrammatic representation of role of phenomics in the genomics era. The figure shows how phenomics in combination with genomics will help us in gene discovery, gene mapping/tagging, cloning and genomics-assisted approaches for crop improvement. *GWAS* genome-wide association study, *PheWAS* phenome-wide association study, *GWS* genome-wide selection, *MARS* marker-assisted recurrent selection, *MAS* marker-assisted selection

plants. These facilities include (i) infrared cameras to scan temperature profiles/transpiration, (ii) fluorescent microscopy/spectroscopy to assess photosynthesis, (iii) three-dimensional camera to record minute changes in growth responses after crop plants are exposed to stresses, (iv) lidars (light detection and ranging) to measure growth rates, and (v) magnetic resonance imaging (MRI) to examine root/leaf physiology (Finkel 2009; Gupta et al. 2012). Digital imaging, considered cornerstone for measuring quantitative phenotypes, has allowed to monitor, measure, and track many aspects of plant development, function, and health which was unimaginable using conventional techniques. A number of software programs have been developed for extracting data from the digital images from roots, shoots, leaves, seeds, grains, etc. A list of software programs and phenotyping platforms for high-throughput precise phenotyping is available elsewhere (see Cobb et al. 2013). These high-throughput phenotyping platforms and software are being used in several laboratories across the world. These phenomics facilities scans thousands of plants in a day and generates huge phenotypic data in the same way as high-throughput DNA sequencing in the field of genomics (Finkel 2009). In some countries, these phenomics platforms are already being used for a variety of phenotypic screens and developing root imaging, for instance, Australian Plant Phenomics Facility in South Australia, Plant Accelerator in Adelaide, Australia; Jülich Phenomics Centre by the Institute for Phytosphere Research (IPR) in Jülich, Germany; and Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben, Germany, to name a few (Mir et al. 2012).

The availability of high-throughput phenotyping tools have although helped in obtaining a large quantity of images and data, but to run the data storage, handling and analysis will be another challenge in plant phenomics. The volume of data mainly depends on the resolution of the image detectors and the number of acquired images from each inspection. The data analysis methods, such as principle component analysis (PCA) (Yang et al. 2009), support vector machine (SVM) (Romer et al. 2011), and artificial neural network (ANN) (Karkee et al. 2009), are often used for data dimension reduction and efficient parameter extraction. In the future for promoting the application of high-throughput plant phenotyping, the less expensive, less laborious, and well-sophisticated data analysis infrastructure, such as HTPheno (Hartmann et al. 2011) and IAP (Klukas et al. 2012) incorporating the open-source software imageJ, needs to be developed and popularized.

Reports are also available on high-throughput phenomics methods/platforms that have been developed and used successfully for targeted trait evaluation in several crop species/plants. For instance, in case of *Brachypodium distachyon*, evaluation for root system architecture under differential nutrient availability was undertaken using high-throughput plant growth and imaging platforms (Ingram et al. 2012). In *Arabidopsis*, plant responses to soil water deficit were dissected using automated visualization and image quantification "PHENOPSIS" platform (Granier et al. 2006). Similarly, high-throughput/high-resolution phenotyping was conducted for measurement of hypocotyl growth and shape (using HYPOTrace), and the seed-germination analysis platform "GERMINATOR" was used for recording data on recombinant inbred lines (RILs) of mapping population, leading to the discovery of several QTLs for salt tolerance (Joosen et al. 2009; Wang et al. 2010). Several other fully or partially automated imaging platforms for non-destructive image-based phenotyping were used to record data in *Arabidopsis* (see Sozzani and Benfey 2011).

In higher plants like rice, automatic and non-invasive imaging system and analysis platforms have been used for accurate phenotyping (Ishizuka et al. 2005). These platforms have been also used to automatically estimate root structure traits (RSA) of 12 rice genotypes, and the results are believed to prove valuable for phenotyping of individuals within mapping populations for identification of genes/QTLs underlying the RSA (Iyer-Pascuzzi et al. 2010). In addition, selected platforms like "PHENODYN" that imposes drought scenarios

have been used to image maize and rice plants for trait evaluation (Sadok et al. 2007). Additional phenomics platforms are being developed for quantification of hyphal growth rates, higher level of automation, and expanding the host and pathogen range (Douchkov et al. 2012). Phenomics platforms have been developed worldwide including Australian Plant Phenomics Facility (APPF; http://www.plantphenomics.org.au/); Jülich Plant Phenotyping Centre (JPPC; http://www.fz-juelich.de/ibg/ibg-2/EN/Research/Phenotyping/Phenotyping_article.html?nn=548814); Laboratory of Plant Ecophysiological Responses to Environmental Stresses (LEPSE; http://www1.montpellier.inra.fr/ibip/lepse/english/), IBERS, Aberystwyth University, UK (http://www.aber.ac.uk/en/ibers/facilities/new_builds_at_ibers/); PhenoPhyte (USA; http://PhenomicsWorld.org/PhenoPhytewebcite); and European Plant Phenotyping Network (EPPN). These platforms have been discussed separately in Chap. 19.

18.5 Phenomics for Harnessing Genomics

18.5.1 Genetic Dissection of Complex Traits

As mentioned above, most of the agriculturally and economically important traits like yield, quality, and some forms of abiotic stress tolerance are controlled by a large number of genes having small effects. These traits are known as quantitative or polygenic traits and sometimes we call them complex quantitative traits owing to their complex genetic control. These complex quantitative traits are highly influenced by the environment, and thus their improvement through conventional breeding methods becomes less rewarding. During earlier times, quantitative traits were dealt by using several statistical procedures based on mean, variance, and covariance and were used to partition the total phenotypic variance into genetic and environmental variance; also, genetic variance was further partitioned into additive, dominance, and epistatic variance. However, little was known about the genes, their location, their effect on a particular trait, and their interactions with each other (epistasis) (Kearsey and Farquhar 1998). With the tremendous advancements in the area of genomics in the recent past, the situation has changed. We can now predict the function, location, nature, and interactions of a gene with maximum certainty. However, the precision of prediction of genes and their functions through genomics needs to be enhanced by making use of high-throughput phenomics tools and technologies. This will help us to harness the full benefits/potential of genomics for crop improvement programs. Meaningful QTL/gene discovery programs either through QTL mapping or association mapping need accurate and precise phenotyping data of complex traits. It is important to mention here that valid and applicable results reported with non-conventional approaches so far have not yielded expected results, in spite of huge molecular genotypic data generated during the last few years (Edmeades et al. 2004; Araus et al. 2008; Collins et al. 2008; Xu and Crouch 2008; Passioura 2010). One important reason is the slow progress in the area of phenomics which involves a number of approaches for recording precise and high-throughput phenotyping data. The phenotypic data is the primary data required for the genetic dissection of quantitative traits and should be taken precisely. To obtain a clean set of reproducible and precise phenotypic data of complex traits like salinity tolerance from larger germplasm collection remains an open challenge even in the era of phenomics-driven technology (Mir et al. 2012). Phenotypic data should be taken with care because these characters are highly influenced by environmental variations and are thus more subjected to experimental errors. The gap between genotype and phenotype is reduced through proper, accurate, and precise phenotyping of quantitative traits (Tuberosa 2012). The precision with which the relevant QTLs or chromosomal regions are identified and their effects are accurately estimated depends on how precisely the phenotyping data are recorded to establish the phenotype-genotype

association (Tuberosa 2012). In other words, the use of molecular approaches for crop improvement depends upon how well and how accurately the targeted trait has been assessed phenotypically in mapping population or diversity panel, because if the phenotypic data is not taken accurately there will be more false positives and false negatives. The genomic approach called GWS that disregards QTL identification relies on the molecular profiling and accurate phenotyping of each progeny (Bernardo 2008; Bernardo and Yu 2007; Heffner et al. 2009). Good phenotyping increases accuracy, precision, and throughput at all levels of biological organization while reducing costs and minimizing labor through automation, remote sensing, data integration, and experimental design (Cobb et al. 2013). "Accuracy" and "precision" are two different terms but often used interchangeably; accuracy involves the degree of closeness of a measured or calculated quantity to its actual (true) value, whereas precision, also known as reproducibility or repeatability, means the degree to which further measurement or calculations show the same or similar results. Most of the quantitative characters show low to moderate heritability, and this low heritability impairs the probability of detecting the presence of QTLs (Bernardo 2004), thereby increasing type II errors (i.e., false negatives). These type II errors can be minimized by taking phenotypic data for the large-sized population, over a number of years, over a number of locations, and thus increasing the probability of detecting the QTLs. MAS based on those QTLs detected by analysis of phenotypic data of a single environment could be inadequate, and due to this situation, the QTL main effect and QE interaction could not be separated. As a result for developing broadly adaptable varieties, new breeding strategies based on QTL evaluation among a variety of environment will be necessary to realize the potential of MAS (Yuan et al. 2006). Simultaneous treatment of phenotypic data from multiple environments provides a significant increase in statistical power of QTL detection and accuracy of the estimates of QTL position and effect (Jansen et al. 1995).

Biparental populations like recombinant inbred lines, doubled haploid population, or diverse germplasm are used to identify QTLs and ultimately clone genes of interest under these QTLs. This process of identification of QTLs involves the use of two types of data: phenotypic and genotypic data. Phenotyping of the populations has been recognized as the most laborious and technically challenging part of this process. Screening of the populations for a valuable agricultural trait (such as biotic stress tolerance, abiotic stress tolerance, grain quality, or yield potential) requires replicated trials across multiple environments over a number of seasons. Tools that are currently in common use for phenotyping require destructive harvests at fixed time intervals or at a particular phenological stage and are slow and costly. Furthermore, if a promising candidate gene is to be tested for allelic variation in a mapping population, this phenotyping work needs to be done precisely. The labor-intensive and costly nature of conventional field phenotyping have meant that many crop breeding programs make a single measurement of final yield for replicated plots in contrasting environments over multiple seasons. However, yield itself is one of most poorly inherited traits in crop breeding. The bottleneck in field phenotyping has driven intense interest over the past decade in applying remote sensing technologies to field crop monitoring, and in this regard field phenomics is more advanced in many respects than controlled-environment, high-throughput analysis. The "phenotyping bottleneck" described above can now be addressed by combining novel technologies such as noninvasive imaging, spectroscopy, image analysis, robotics, and high-performance computing. Phenomics could be described as simply "high-throughput plant physiology." As a result, field evaluation of plant performance is much faster and facilitates a more dynamic, whole-of-life cycle measurement less dependent on periodic destructive assays. Furthermore, application of these tools in dedicated high-throughput, controlled-environment facilities has the potential to improve accuracy and precision and reduce the need for replication in the field.

18.5.2 Gene/QTL Discovery

Above we have discussed the role of phenomics to dissect the complex traits using genomic resources. Both forms of linkage mapping have been used successfully to identify the genomic regions underlying the complex traits. However, cloning of the gene(s) underlying the QTL remains time-consuming and resource intensive, although QTL explains a substantial proportion of the phenotypic variation (Bhattacharyya 2010; Fan et al. 2006; Krattinger et al. 2009; Li et al. 2010; Liu et al. 2008; Saito et al. 2010). The poor phenotyping of targeted trait increases the rate of discovery of false QTL. Therefore, advances in phenomics have potential to improve accuracy and precision of phenotyping. As a result, both forms of linkage mapping hold great promise for elucidating the genetic architecture of complex traits and identifying the genes and specific alleles underlying trait variation. In barley, transient-induced gene silencing (TIGS)-based phenomics platform has been developed and used for testing of ~1,500 genes. The analysis revealed identification of 70 candidate genes showing significantly increase in resistance or susceptibility to fungal disease upon TIGS. Besides this, high-throughput phenomics platforms/tools have been used for genetic dissection leading to discovery of genes/QTLs for several traits including root architecture traits, seed shape, osmotic tolerance, and biomass traits in crops like rice, wheat, barley, and mustard (Tanabata et al. 2012; Shi et al. 2012; Topp et al. 2013). These successful examples suggest that phenomics holds great promise in uncovering all the useful genes/QTLs governing complex quantitative traits in plants.

18.5.3 Identification of Gene Function

During the past two decades, our understanding of genotype has improved manifold due to availability of millions or billions of nucleotides. This has helped to determine genotypes down to the level of individual nucleotides in whole genome. In addition to this, other genomic tools and techniques have made it possible to characterize the natural genetic variation at routine basis and to support trait-driven efforts to clone and understand specific genes. Now, genome science has been moved beyond the era of reference and model organisms leading to identification of candidate genes for targeted traits (Cocuron et al. 2007). However, the function of several genes is still unknown due to the incomplete knowledge of all the expressed traits of the individual. Therefore, high-resolution and high-throughput technologies of plant phenomics are required for exploiting the wealth of gene sequence information provided by the "genomics revolution" and mine agricultural germplasm for genetic diversity. The phenotypic database similar to genomic database can help to know the unknown function of gene sequences following the reverse genetic approaches of genomics. Phenotypic profiling of a mutant and the wild-type plant for growth rate, growth behavior, timing of flowering and seed set, seed shape, leaf shape, color changes, root density, nutrient utilization, and other deviations can generate vast amounts of data for systemically studying the relationship between them. The integration of phenomics data with data sets from transcriptome, proteome, metabolome, and genome sequences will help to paint a comprehensive picture of mutant gene function.

18.5.4 Precision Phenotyping for Genomic Selection

Large-scale, rapid, and simple precision phenotyping is significant for breeders engaged in variety development following the genomic selection. In recent years, the cost and efficiency of obtaining genomic information on large numbers of individuals have been drastically reduced compared to collecting the phenotyping data over years and environments. Therefore, breeding community has focused more on genomics selection for predicting phenotypic outcomes

(Cabrera-Bosquet et al. 2012; Heffner et al. 2009; Heslot et al. 2012).

Besides, the use of Bayesian models facilitates the analysis of limited individuals for phenotypic performance sparse data (where not all individuals or families are evaluated phenotypically in each environment). This has also encouraged the use of genomic selection. If genomic selection demonstrates a clear increase in the rate of genetic gain per cycle of selection, then breeders will quickly adopt the most efficient strategy to accomplish their goals. For this purpose, breeders are required to use the cost-effective, accurate, and highly precised phenotyping tools for genomic selection. Thus, phenomics tools can help to make genetic improvement in a targeted trait through genomic selection. Moreover, next-generation phenotyping tools and techniques can provide the precise phenotyping data of a trait on a training population and thus allow geneticists/breeders to dissect the complex traits into genotype, phenotype, and the environment. This phenotyping data can be used to develop models that leverage genotypic information to predict phenotypic outcomes. Precision phenotyping is most important for developing the genomic selection model because phenotyping database obtained from training population provides the basis for developing the statistical model. This genomic selection-based statistical model is then used to predict phenotypic performance in related members of a breeding population (see Cobb et al. 2013 for details).

18.6 Conclusion

Exploitation of the potentiality of available germplasm resources by using the genomics approaches for development of improved genotypes in crop plants requires a better understanding of physiology and genetic basis of important traits related to yield, quality, and biotic and abiotic stresses. Therefore, an accurate and cost-effective phenotyping is important. In recent years, new developments have been made in the field of phenomics for screening the traits more accurately and precisely. These advances have facilitated the development of high-throughput phenotyping platforms for screening the germplasm. Utilization of techniques/approaches improved the collection of phenotypic data more precisely and cost-effectively with reduced experimental noise. Thus, accurate phenotyping will help to estimate the real heritability of a trait, which is essential to make genetic improvement through genomic selection. As a result, genomic resources can be utilized with full potential by identifying the real QTL for complex traits, in discovery of genes under the QTL and identification of function of those gene sequences which are still not known. In the future, the phenotyping facilities will be available to plant breeders which will be utilized routinely to screen large population for making genetic improvement in crop plants by precise manipulation of genes through genomics.

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High-Throughput Plant Phenotyping Platforms

19

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Abstract

To meet the ever-increasing demand of food and feed for the burgeoning population, we need to double our food production by 2050 with a growth rate of about 2.4 %. This needs input-responsive, resource-use-efficient and short-duration genotypes which are stable and can perform well in an array of situations. For this, integrated breeding efforts connecting genomics and phenomics together are required. While a giant leap has been made in crop genotyping in the last two decades, especially with the development of next-generation DNA sequencing, the latest developments in automation, robotics, accurate environmental control and remote sensing facilities have offered opportunities for precise field phenotyping of crop plants through state-of-the-art high-throughput plant phenotyping platforms (HTPPs). Although the initially developed platforms had limitations with regard to accuracy, speed and ground clearance, the latest HTPPs are capable of taking multiple trait measurements simultaneously that have improved data acquisition as well as provide high-throughput phenotypic data required for crop breeding programmes. A number of analysis pipelines have also been developed which are equipped with high-speed computing. This chapter describes some of the most popular HTPPs and their specific features to achieve precision phenotypes in crop plants.

Keywords

Biotron • High-throughput phenotyping • HTPheno • HTPPs • Image analysis • Phenotyping • RSA • PHENOPSIS • PhenoFab

19.1 Introduction

Crop performance and ultimately its production depend upon the quality of seed, its agricultural management and the environment. Plant breeders

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always aim at the development of a variety which is characterized by improved input (nutrition and water)-use efficiency. With the ever-increasing food demand of the world, the exploitation of costly natural resources including water is bound to increase, and therefore, this requires a comprehensive management of the crops and monitoring of resource use across the whole food chain (Gebbers and Adamchuk 2010). The human population is likely to exceed nine billion by 2050 which will pose tremendous challenges to ensure sufficient and nutritious food supplies for all. Achieving this goal will especially be a challenge to plant breeders because crop yields will need to grow at a rate of 2.4 % annually, even though the current growth rate is 1.3 % with yield stagnating in up to 40 % of the land under cereal production (Ray et al. 2013). Declining factor productivity, stagnation in net cultivated area and an array of biotic and abiotic stresses further pose additional constraints in increasing the productivity of crop plants. Of late, climate change has emerged as another potential threat which may manifest itself in the form of droughts, floods, tropical cyclones, temperature extremes and heat waves which are known to negatively impact agricultural production. The projected increase in these events will result in greater instability in food production and threaten livelihood security of farmers (Singh et al. 2013).

Over the last two decades, considerable development has been made in the genomics of crop plant. However, efficient phenotyping to connect genotype with phenotype remains a constraint. Efforts of integrating new molecular tools, especially in dissection of complex quantitative traits, are seriously hampered by our limited ability to phenotype the plants precisely and efficiently. Therefore, a fundamental step forward is to dramatically improve the phenotypic prediction based on the genetic composition of lines or cultivars (White et al. 2012). A continuous flow of advances in the phenotyping techniques available to breeders offers the potential to increase the rate of genetic improvement (Phillips 2010). Nevertheless, phenotype-based selections have been continuously made by farmers and breeders long before the systematic breeding efforts were initiated. Plant breeding is essentially a number game and it largely depends upon the visual selection of the best genetic variation in a (segregating) population. However, to meet the demands of future food needs as well as its quality, a geometrical enhancement in breeding efficiency is required which can be achieved by integration of molecular and conventional approaches of crop improvement. Plant Breeders want to be able to phenotype a large number of lines rapidly and precisely to identify the best variant in his population. This requires precise high-throughput phenotyping, especially of the complex traits.

Accurate phenotyping is necessary not only in conventional crop improvement programmes but also for genomics-assisted breeding. Although molecular breeding strategies, such as marker-assisted recurrent selection and genomic selection, place greater focus on selections based on genotypic information, they still require phenotypic data (Jannink et al. 2010). Similarly, phenotypes are also required to train a prediction model in genomic selection (Lorenz et al. 2011). In transgenics also, phenotyping is required to identify promising events (Gaudin et al. 2013; Saint-Pierre et al. 2012). Advances in high-throughput phenotyping have provided fast and inexpensive genomic information (Araus and Cairns 2014). Realizing the importance of phenotyping of crop plants, advances in high-throughput phenotyping have provided several root and shoot phenotyping platforms which will be described in this chapter.

19.2 High-Throughput Phenotyping

Precise phenotyping under field conditions still remains a major bottleneck in most of the varietal improvement programmes (Cobb et al. 2013; Araus et al. 2008; Cairns et al. 2012). Field conditions are highly heterogeneous with little or no control over the environmental factors. On contrary, experiments in the controlled environments, such as in greenhouse, are far from the real situations in the field and therefore

cannot be easily extrapolated in real situations (Araus and Cairns 2014). For example, soil which is the most crucial factor in plant growth and development, supplying essential nutrients and water, always remains variable in field conditions. Similarly, in controlled conditions in pots, the volume of soil may be too less as compared to the field conditions and may not mimic the real world situation. Also, plants are generally grown in isolation in pots under controlled conditions in comparison to field where these are grown in a plot or cluster, having a number of plants in the periphery, and therefore, may lead to a crucial difference in the canopy development. Most of the varietal development programmes rely largely on multilocation evaluation, and in many such programmes, some of the environmental variables are not monitored properly and hence are poorly understood (Araus and Cairns 2014; Cairns et al. 2013). This further complicates the ability to mimic the field environment under controlled conditions. Similarly, many stresses go unnoticed, except for the major stresses or those which are targeted at for evaluation, and, therefore, are generally reproduced in the artificial phenotyping conditions. Objectives of phenotyping, heritability of the trait under consideration and available logistics also influence the method of phenotyping.

High-throughput phenotyping platforms (HTPPs) are fully automated facilities encompassed in growth chambers or greenhouses and supported by robotics, precise environmental control and remote sensing facilities to assess overall growth and development of the plant. Development of HTP systems for plants has mostly focused on measuring traits of individual plants in environmentally controlled chambers or greenhouses. However, many of the agronomically important traits are best expressed when the plants are grown in population under the relevant edaphic and environmental conditions. Therefore, there is an increased interest among researchers in field-based HTPPs. There are a number of vehicle-based high-throughput systems used for phenotyping of crop plants in the fields. Initially, deployment of single sensor-type platforms was proposed. For example, a three-wheeled cart was used by Ruixiu et al. (1989) to position multiple ultrasonic sensors around a single row of a crop. Montes et al. (2007) proposed tractor- or harvester-mounted reflectance sensors for collection of spectral data. Later, McCarthy et al. (2010) used a machine vision system to measure internode length in cotton. However, the major restriction with these systems was the limitation in measuring multiple traits, which is needed most of the times in typical crop breeding experiments. To take measurements on multiple traits, Lan et al. (2009) devised a system which has integrated sensors for leaf area index (LAI), crop canopy height, NDVI, multiple spectral imaging and hyperspectral reflectance. Comar et al. (2012) used a platform which had a number of instruments including a global positioning system (GPS), an antenna, an irradiance probe, a digital camera and a passive spectrometer. Similarly, White and Conley (2013) used multiple sensors mounted over a handcart which could position the sensors over two rows. However, it required continuous labour to move the carts and also had clearance limitations. These systems definitely provided opportunities for improving the acquisition of phenotyping data, but these were not capable of providing actual high throughput needed for modern varietal development programmes. To improve high throughput, Andrade-Sanchez et al. (2014) used a phenotyping system carrying four sets of sensors to measure canopy height, reflectance and temperature simultaneously on four adjacent rows of cotton (*G. barbadense* L.) and were able to collect phenotypic data at a rate of 0.84 ha/h.

19.3 Imaging and Analysis Platform for Plant Root Systems

Plant health and survival are dependent on the plant root system architecture (RSA) (Iyer-Pascuzzi and Sozzani 2014) as well as the spatial configuration of different types and ages of roots emerging from a single plant (Lynch 1995). Root systems are complex plant structures that have an important role in certain plant functions

including nutrient and water acquisition. Therefore, RSA and its development have received increased attention due to advances in phenotyping capabilities and growing insights into the genetic control of root growth (Tuberosa and Salvi 2006; Armengaud et al. 2009). While a number of external factors can affect the morphology and architecture of roots, certain root qualities in crop plants help them in their stability in problem environments and also in enhancing their productivity in resource-limited conditions. Therefore, introgression of intrinsic and environmentally responsive root architectural characters into crop plants may help combat environmental vagaries and result in enhanced productivity in stress conditions.

Extreme variability and complexity of field environments and high responsiveness of root systems make it difficult to obtain precise information on the genetic components of RSA and developmental root traits under field conditions. Therefore, elucidating the genetic and developmental basis of RSA in natural conditions presents many challenges that must be addressed through a combination of field-, greenhouse- and laboratory-based approaches (Clark et al. 2011) as well as predictive practices. In situ methods involving rhizotron, magnetic resonance and computed tomography have been developed to facilitate non-destructive spatial and temporal investigations into the root system grown in soil (Gregory et al. 2003; Tracy et al. 2010). In addition, simulation and modelling studies linking rhizosphere and growth data help linking the predictive and field studies. Techniques such as gellan gum growth systems with superior optical clarity have been developed to facilitate non-invasive two-dimensional (2D; Iyer-Pascuzzi et al. 2010) and three-dimensional (3D; Fang et al. 2009) imaging and temporal studies of plant root systems. One such high-throughput RSA phenotyping platform using RootReader3D Reconstruction and Analysis Software provides a capacity to measure root traits with high degree of spatial and temporal resolution and facilitates novel investigations into the development of the entire root systems or the selected components of the root systems (Clark et al. 2011). The 3D imaging and RootReader3D software is a unique imaging and analysis platform for investigating both static and dynamic 3D RSA characteristics of plant root systems. The software RootReader3D utilizes a silhouette-based back projection algorithm (Mulayim et al. 2003; Zhu et al. 2006) combined with cross-sectional volume segmentation to generate 3D root models (for details see Clark et al. 2011). To assist in visualizing and interacting with the 3D reconstructions and automated analysis of RSAs, viewing interfaces and commands have been incorporated into the RootReader3D software. In general, this platform provides an opportunity of enhanced quantification capabilities and capacity to image over 100 root systems per day and presents many opportunities for dissecting the genetic control and developmental changes of RSA as well as opportunities to explore RSA variation within and between species grown in a range of environmental conditions.

19.4 Field-Based High-Throughput Phenotyping Platforms

A combination of advances in aeronautics, remote sensing and high-performance computing has paved the way to develop ground-based platforms to aerial systems of HTPPs (Araus and Cairns 2014). Most of the ground-based HTPPs include modified vehicles equipped with GPS, navigation devices, cameras and sensors (Araus and Cairns 2014). The ground-based HTPPs enable the data to be captured at the plot level and do not require much post-data processing. However, due to limitations in ground-based HTPPs, it is not possible to take measurements in all plots simultaneously. The other category is of aerial HTPPs which can be used as a solution to the problems associated with the ground-based HTPPs. The initial aerial HTPPs employed small aeroplanes to take measurements which used to be a costly affair. Further, many times it is also not possible to achieve the desired speed and altitude to take high-resolution images. The recent alternatives to aeroplanes in aerial HTPPs

include 'phenotowers' (Rascher et al. 2011) and 'blimps' (Losos 2013). These also have limitations such as they require many people to handle and a large space for storage. The alternatives to 'blimps' are unmanned aerial platforms (UAPs) such as polycopters. UAPs although carry less amount of weight, they enable greater flight control and autonomy and are comparatively more affordable. Most of the UAPs carry a camera together with a thermal imaging sensor.

19.5 High-Throughput Phenotyping Platforms

In the past two decades, precision agriculture has emerged as a major discipline for the most optimum and integral use and management of natural resources. Of late, automation, robotics, high-speed computing, new sensors and imaging technologies have made available a range of applications for laboratory research and screening systems for horticultural and production systems (Belforte et al. 2006; Grift et al. 2008; Zude 2009; Fiorani and Schurr 2013). Consequently, plant phenotyping has also changed into precision phenotyping which requires HTPPs. A number of automated plant phenotyping platforms have been developed during the last one and a half decade. These are capable of large-scale phenotyping, although restricted to a few select species such as *Arabidopsis* (Arvidsson et al. 2011; Granier et al. 2006; Skirycz et al. 2011), cotton (Andrade-Sanchez et al. 2014) and some of the major cereals (Golzarian et al. 2011; Hartmann et al. 2011).

Most of the HTPPs currently in operation throughout the world are fully automated, high-speed platforms housed in greenhouses or growth chambers. These platforms utilize robotics, precise environmental control, remote sensing techniques, global positioning systems and high-speed computing facilities to assess vital parameters of plant growth and development and dissect their genetics. These HTPPs are mostly run by large seed companies and advanced crop research institutes around the world. Some of the popular HTPPs around the world include PHENOPSIS, *Arabidopsis* platform, INRA (http://bioweb.supagro.inra.fr/phenopsis/InfoBDD.php); PhenoFab, Wageningen (KeyGene + LemnaTec) (http://www.phenofab.com/); Biotron, Canada (http://www.thebiotron.ca/); IBERS, Aberystwyth University (http://www.aber.ac.uk/en/ibers/n); LemnaTec (http://www.lemnatec.com/); International Plant Phenomics Network (http://www.plantphenomics.com/) integrating a few of the national HTPPs; Jülich Plant Phenotyping Centre (JPPC) (http://www.fz-juelich.de/icg/icg-3/jppc); LEPSE, Montpellier (http://www1.montpellier.inra.fr/ibip/lepse/english/); Australian Plant Phenomics Facility (http://www.plantphenomics.org.au/); Smart tools for Prediction (and) Improvement of Crop Yield – peppers (SPICY) (http://www.spicyweb.eu/) and PPHD, INRA, Dijon (http://www.dijon.inra.fr/dijon_eng/toute_l_actu/journees_internationales_de_limnologie).

The most successful traits for evaluation integrate in time (throughout the crop cycle) and space (at the canopy level) the performance of the crop in terms of capturing resources (e.g. radiations, water and nutrients) and how efficiently these resources are used (Araus and Cairns 2014; Cairns et al. 2012). These traits are evaluated using different approaches, viz., (1) proximal sensing and imaging, (2) laboratory analysis of samples and (3) near-infrared reflectance spectroscopy (NIRS).

19.5.1 PHENOPSIS

PHENOPSIS is a publicly available (URL: http://bioweb.supagro.inra.fr/phenopsis/) information system developed for storage, browsing and sharing of online data generated by the PHENOPSIS platform and offline data collected by experimenters and experimental metadata (Fabre et al. 2011). This system was developed by Granier et al. (2006) for reproducible phenotyping of *Arabidopsis thaliana*. It is a prototype built by Apilogic (Fondettes, France) and

is composed of a steel frame supporting 14 trays. Each tray has 36 holes which can support a pot and a mechanical arm able to move according to a program developed by Apilogic on APIGRAPH IP software. The arm can be loaded with displacement sensors, balance, camera and tube for irrigation. The experiment comprising different components, viz., position of pots on the trays, time and cycle of irrigation schedules, time of taking a picture, etc., can be easily programmed in a computer on APIGRAPH IP software. The climatic regulation of the growth chamber can also be controlled by a computer with the software, and the computer in turn can be connected with various kinds of sensors, viz., light, humidity, leaf temperature, etc., helping in the measurement of each micrometeorological condition. In platforms such as this, large quantities of environmental data, plant images and phenotypic data are produced for the study of genotype X environment interaction effects on different plant processes (Fabre et al. 2011). Nevertheless, this system requires procedures for proper handling of datasets, their extraction and sharing with the scientific community.

19.5.2 PHENOPSIS DB

PHENOPSIS DB provides a solution to the storage (database), analysis and sharing (web interface and web services) of images and data collected in the PHENOPSIS platform (Fabre et al. 2011). This platform provides modules coupled to a web interface for (1) the visualization of environmental data of an experiment, (2) the visualization and statistical analysis of phenotypic data and (3) the analysis of *Arabidopsis thaliana* plant images. The database was developed using the MySQL 5.0 Community Server, while the Web interface was developed using XHTML, PHP, JavaScript, jQuery, Ajax and CSS. The RODBC package in R version 2.9.2 was used to establish the database connection.

All metadata on PHENOPSIS DB can be accessed freely. The metadata include characteristics of experiments and associated protocols, genotype information in an experiment, list of different variables under consideration in an experiment and associated protocols, micrometeorological data and comments on experiments. The major advantages and utilities of PHENOPSIS DB include that this is a user-friendly Web interface and has an interoperability with other databases. Users of this system can download and analyse the publicly available datasets, environmental conditions during an experiment and various images related to an experiment besides consulting various experiments and genotypes.

19.5.3 Phenoscope

It is a platform having a unique feature of continuously rotating 735 individual pots over a table, adjusting watering automatically. It comprises of an aluminium table on a steel structure maintained in a growth room. On this structure, up to 735 plants may be grown and phenotyped individually. The table is equipped with weighing and watering arrangements so that the plants can be maintained at a given treatment target. The table contains a closed-circuit track and a series of buttons to hold the pots. The individual pots, each designed to hold a single plant, are pushed along the guiding rails by coordinated pusher arms that allow the robot to move each pot sequentially across all possible positions on the table in a single cycle, up to six times every 24 h (Tisn et al. 2013). The movement of the plants is designed in such a way that every individual plant despite the environmental heterogeneity experiences the same environmental conditions on an average over time which is not on a comparative advantage or disadvantage. Non-destructive phenotyping can be performed at the phenotyping station available at the right-hand corner of the table. A digital camera takes images of the plants on the Phenoscope and labels and stores them on an image server for further retrieval and analysis. The Phenoscope makes it possible to perform large-scale

experiments that would not have been possible or reproducible by hand (Tisn et al. 2013).

19.5.4 HPGA

HPGA (https://www.msu.edu/~jinchen/HPGA/) is an HTPP for plant growth modelling and functional analysis (for details see Tessmer et al. 2013). It has two components, plant area estimation (PAE) and growth modelling and analysis (GMA). In PAE, by taking the complex leaf overlap problem into consideration, the area of every plant is measured from top-view images in four steps. In GMA, a nonlinear growth model is applied to generate growth curves, followed by functional data analysis.

In most of the high-throughput computational phenotyping techniques, top-view images are captured periodically, and consequently a growth curve is generated using the observed value of a plant over time (Zhang et al. 2012). However, the observed value of a plant from top view is affected by leaf overlap as well as leaf twisting, curling and circadian movements, which result in inaccurate estimates of growth patterns. This problem becomes more severe with older plants having too many larger and overlapping leaves. HPGA estimates the leaf overlap percentage to measure plant area more accurately. Unlike the existing techniques that simply count the number of valid pixels in an image (Harris 1989; Heinen 1999), HPGA estimates plant areas accurately through a four-step approach: plant centre identification, leaf tip identification, leaf area estimation and plant area measurement (Tessmer et al. 2013). In HPGA, functional data analysis is applied on growth curves for better interpretation of the plant growth scenarios. This approach avoids the leaf segmentation problem to recognize all the leaves of a plant from a top-view image. With this high-throughput phenotyping technique, researchers are able to generate hundreds or even thousands of observations for every plant automatically. However, the major limitation with this model is that the leaf-to-area model is genome specific, and changing from one species to another needs to train the model again with the new leaves.

19.5.5 The Plant Accelerator

The Plant Accelerator (http://www.plantphenomics.org.au/services/accelerator/?template=print) combines the digital imaging technologies, high-capacity computing and robotics for automated, high-throughput, non-destructive phenotyping of crop plants. It is situated in a customized facility, which comprises most modern greenhouses, growth rooms, laboratories and seed storage facilities. Located at the University of Adelaide's Waite Campus and supported by the Australian Government under the National Collaborative Research Infrastructure Strategy (NCRIS) and the University of Adelaide, the Plant Accelerator provides users an expertise in plant and soil science. Research projects facilitated by this technology vary from large-scale screening of early growth, salinity tolerance to water- and nutrient-use efficiency.

This HTPP increases the speed and accuracy of plant physiological measurements. It also helps in carrying out projects with large populations of plants enabling genetic studies to be undertaken to identify the molecular basis of complex physiological traits besides providing a better understanding of how the environmental components affect plant growth and performance.

The Plant Accelerator uses imaging stations (LemnaTec Scanalyzer 3D) for the non-destructive phenotyping of plants. This system consists of visible light images (RGB) which allow the measurement of shoot area and inferred mass, plant height and width, canopy density, other morphometric data, leaf colour and senescence. Further, steady-state fluorescence imaging with blue light large field excitation (<500 nm) allows quantification of plant senescence, chlorosis and necrosis and programmable watering to weight of plants to enable large-scale experiments requiring controlled watering levels.

19.5.6 Biotron, Canada

The Biotron (http://www.thebiotron.ca/) is also a multidisciplinary, experimental climate change research centre situated at the University of Western Ontario, Canada. It is a unique, purpose-built facility having specialized environmental chambers, laboratories and equipment. Biotron facilities include separately contained research modules for earth sciences, microbiology, plants (including transgenics), algae and cyanobacteria and insects. Within these modules are laboratories and equipment providing custom-designed controlled environments and analytical tools. Most of the Biotron's equipments can be controlled or monitored over the internet from any geographical location and the remote users are helped by technicians and specialists available within the Biotron. The Biotron is equipped with an Imaging and Data Analysis Suite, Analytical Laboratory Suite as well as a Flow Cytometry Suite, with their associated laboratories and imaging facilities. The Biotron enables the integration of experimental climate change and environmental research from the molecular scale to the mini-ecosystem scale. Many instruments, growth chambers and imaging devices are networked to a secure, remotely accessible central server enabling global access.

19.5.7 LEPSE (Ecophysiology Laboratory of Plants Under Environmental Stress), Montpellier

This facility located at Montpellier (http://www1.montpellier.inra.fr/ibip/lepse/english/) is a state-of-the-art phenotyping facility with the capacity of high-throughput analysis of transpiration, leaf expansion, reproductive development, canopy architecture as well as soil water content, apex temperature and other micrometeorological variables. Further in situ measurement of cell turgor, hormones and kinematic analysis of cell division rate and tissue expansion rate is also possible in this facility. The phenotype is considered via time courses of organ expansion, plant transpiration and 3D development of leaf surfaces, involving several plants in different experimental contexts.

19.5.8 PhenoFab, Wageningen (KeyGene + LemnaTec)

The PhenoFab™ (http://www.phenofab.com/) is a high-tech phenotyping platform housed in a greenhouse set-up. In this platform, determination of the phenotype is based on moving pots or trays with plants through a greenhouse compartment and fixed scanning areas containing image technology to capture digital data. The plants grow in individual containers/tray wells and are scanned at preset time points from various angles using VIS (visible light), NIR and fluorescent imaging technology. The facility has climate-controlled compartments, enabling experiments with different growth or stress factors. Digital trait analysis is based on visible light and NIR (near-infrared spectrum) imaging as well as fluorescent imaging. RGB images are analysed using pixel values to identify shape, colour and other morphological digital phenotypes. The NIR-infrared images are specifically employed to reveal internal structures of plants, water content or other (e.g. chemical) compositions. The fluorescence imaging allows GFP-protein and chlorophyll analysis to be performed. Combinations of all imaging technologies can be used to resolve objects that have low variability in any one spectrum.

19.5.9 High-Throughput Phenotyping Platform (PPHD), INRA

The PPHD platform (http://www.dijon.inra.fr/en/Tools-and-Resources/Tools_resources_dijon_inra/PPHD) is composed of adjustable greenhouses, air-conditioned chambers equipped with conveyors and high-throughput phenotyping chambers complete with robots and cameras for studying the aerial and root sections

of thousands of plants, on various wavelengths. This platform allows various stages of plant growth and development to be studied, along with physiological functioning in closely monitored conditions. This technique allows the behaviour of thousands of representative and genetically diverse plant species to be analysed in controlled cropping conditions, in order to identify the most suitable species. Phenotyping thus allows plant performance to be measured in great detail, and complementary information on plant genes is provided by genotyping.

19.5.10 The Australian Plant Phenomics Facility

Digital imaging technologies, high-capacity computing and robotics are combined at the Australian Plant Phenomics Facility (http://www.plantphenomics.org.au/) to allow the automated, high-throughput, non-destructive measurement of plant growth and function ('phenomics'). These resources are situated in a purpose-built facility, which also houses state-of-the-art greenhouses, growth rooms, laboratories and seed storage facilities.

19.5.11 LemnaTec

LemnaTec phenotyping systems (http://www.lemnatec.com/) are stationary portal crane systems, which can move a measurement support platform in a height of 3–6 m over a field area of several hundred square metres. The platform is moved in a reproducible way with high precision to different positions, where visible light, near-infrared and infrared cameras take images of the field from the top. 3D scanners can add a third dimension to the camera information. All components are housed and equipped with cooling options, so that the system may even be used in tropical and subtropical regions. For analysing competitive systems with a similar attention to detail and depth, this system does not have any technical or measurement limits.

19.5.12 International Plant Phenomics Network

The International Plant Phenomics Network (http://www.plantphenomics.com/) connects scientific institutions and companies related to research in plant phenomics from all over the world. Currently its partners include the phenotyping laboratories in Australia, France, Germany and Canada, including the Australian Plant Phenomics Facility, Ecotron, Centre National de la Recherche Scientifique (CNRS), Ecophysiology Laboratory of Plants Under Environmental Stress (LEPSE), INRA, Jülich Plant Phenotyping Centre (JPPC) and Biotron Experimental Climate Change Research Facility (Biotron).

It aims at developing, integrating and providing novel technologies to analyse plant phenotypes, providing quality assurance measures in the technologies used for plant phenotyping, identifying gene functions and their relationship to environmental cues and analysing the effect of environments on plant structure and function as well as performance of plants in specific environments in the laboratory and in the field. This network also promotes development of new concepts on the interaction between plants and their environment and transfer of novel technologies into applications in plant production and for the analysis of natural plant and ecosystem performance. The major targets of the International Plant Phenomics Network are breeding plants for the changing environment, prognosis of plant performance in global change, innovative plant production for present and future crops based on the understanding of the complex interaction of plants with their environment and its dynamics, monitoring of plant performance in natural systems and providing a science-based concept and technology to address major challenges of plant performance.

19.5.13 HTPheno: Image Analysis Pipelines

Analysing images from high-throughput phenotyping experiments is often time-consuming and demands high-end computational skills. In a single high-throughput experiment, thousands of images are produced. There are several tools which support image editing, image processing and image analysis for many biological applications (Walter et al. 2010). HTPheno, an open-source image analysis pipeline, supplies a flexible and adaptable Image plug-in which can be used for automated image analysis in high-throughput plant phenotyping and therefore derives new biological insights (Hartmann et al. 2011). HTPheno is such a plug-in which provides an adaptable image analysis pipeline for high-throughput phenotyping (Hartmann et al. 2011). It has two built-in functions, (1) calibration (HTPcalib) to specify different parameters for segmentation and (2) automatic image processing, which can be used for analysing colour images from the side view as well as top view. This plug-in realizes automatic image processing for a number of images, and finally, the results of analysis are consolidated in tabular form and the processing steps for each plant may be visualized in an image stack.

19.6 Conclusions

If the crop yields are to double by 2050 to achieve the production goals, the crop improvement has to witness an unprecedented increase in productivity as well as improved resource-use efficiency. This becomes increasingly important in the current scenario of decreasing land and water resources, emerging challenges due to the impact of climate change and decreasing factor productivity. Development of input-responsive, short-duration and high-yielding crop varieties will require a better understanding of the vital parameters of crop growth and development and a dissection of their genetic traits which will be possible through a comprehensive assessment of plants' structural parameters including their growth and development, architecture, physiology, ecology, tolerance/resistance to stress, yield and an array of some of the more complex traits. Unfortunately, the capacity to undertake precision phenotyping is currently not up to that level which can match with the developments occurring in plant genotyping. This creates major hindrances in breeding trait-specific cultivars as well as identification of most vital root and shoot characteristics which could indicate a plant's response to input-use efficiency, stress conditions as well as changing environments. The modern crop breeding requires precise phenotyping of appropriate traits. During the last decade, progress has been made in the development of several HTPPs which have made it possible to come out of some of these bottlenecks, although a major limitation still remains with respect to real-time integration and assessment of data generated. Cost-effectiveness is another issue which remains associated with the development and use of the HTPPs. Therefore, there is a strong need for the development of cheaper but effective and accurate solutions to precision phenotyping. It must be realized that precision phenotyping is not just studying the physiology or genetics of plants but it requires an interdisciplinary approach. The underutilized phenotyping techniques need to be appropriately integrated into regular field phenotyping programmes. For example, there are well-established non-invasive techniques to target seed traits as well as their composition which still remain underutilized. Similarly phenotyping of root traits and their architecture will be most crucial for studying resource-use efficiency, and fully automated newer technologies which can lead to automatic reconstruction of 3D images will be required. Keeping in view the fast-changing scenario of crop research globally, capacity enhancement of the existing and the newly proposed phenotyping platforms will be one of the most important criteria which will decide the success of our future crop breeding programmes and bridging the gap between the genome and phenome.

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