J. Crop Sci. Biotech. 2016 (JUNE) 19 (2) : 177~187
DOI NO. 10.1007/s12892-016-0005-8

RESEARCH ARTICLE

Morpho-Physiological Parameters Associated with Iron Deficiency Chlorosis Resistance and Their Effect on Yield and Its Related Traits in Groundnut

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Received: January 22. 2016 / Revised: March 14. 2016 / Accepted: April 18. 2016

Abstract

Iron deficiency chlorosis (IDC) causes a significant reduction in yield of groundnut grown in calcareous and alkaline soils in India. The main aim of the study was to assess genotypic differences for morpho-physiological parameters associated with IDC resistance across different stages and their effect on yield and its related traits. The factorial pot experiment was comprised of two major factors, i) soil-Fe status [normal-Fe, deficit-Fe], and ii) genotypes [five] with differential IDC response, constituting 10 treatments. They were assessed for five morpho-physiological parameters associated with IDC resistance across five crop growth stages and also yield and its related traits. Associations between these traits were also estimated. Under deficit-Fe conditions, IDC resistant genotypes recorded significantly lower visual chlorosis rating (VCR), higher SPAD values, active Fe, chlorophyll content, peroxidase activity, and high yield compared to susceptible ones. Between normal- to deficit-Fe soils, resistant compared to susceptible genotypes showed no change in VCR scores; a lower reduction in SPAD, chlorophyll, active Fe, peroxidase activity, and pod yield. Under deficit-Fe conditions, high yield among resistant genotypes could be attributed to higher seed weight, number of pods and haulm yield, while contrasting reduction in main stem height and number of primaries. The results indicate that for initial large-scale screening of groundnut genotypes for IDC resistance, SPAD values are most ideal while active Fe could be utilized for confirmation of identified lines.

Key words : iron deficiency chlorosis, groundnut, morpho-physiological parameters, peanut, yield-related traits

Introduction

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop grown worldwide on 25.41 million ha with a production of 45.65 million tonnes. In India, groundnut (5.25 m ha, 9.47 m t) is the second most important oilseed next to soybean (12.2 m ha, 11.95 m t) (Faostat 2013). Iron plays an important role in photosynthesis, respiration, nitrogen fixation, DNA synthesis, hormone production, chlorophyll formation, and is also a component of various redox and iron-sulphur enzymes (Zheng 2010). Iron deficiency chlorosis (IDC) is common world-wide among crops grown in calcareous, alkaline, coarse textured, eroded, and low organic matter-containing and cold region soils as iron is less available for uptake in these soils. Iron deficiency is a problem in most calcareous soils and they are widespread with an estimated 800 m ha worldwide, mainly concentrated in areas with arid or Mediterranean climates (Land FAO and Plant Nutrition Management 2000). High pH and bicarbonate ion concentration in calcareous soils leads to IDC by suppressing iron uptake and/or translocation in plants (Li-Xuan et al. 2005). Other factors appears to be associated with IDC include low temperature, high relative humidity, and high nitrate concentrations in the soil. Iron deficiency is a complex disorder and occurs in response to multiple soil, environmental, and genet-

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ic factors (Wiersma 2005).

Plants adopt two types of mechanisms (Strategy I and II) for iron acquisition from the soils. Strategy I is found among dicots and monocots, except graminaceous species which adopt Strategy II. The Strategy I mechanism involves proton release at the rhizosphere that lowers the pH of soil solution and increasing solubility of Fe^{3+}, Fe(III) chelate reductase activity that reduces Fe^{3+} to more soluble Fe^{2+}, and transportation of Fe^{2+} into the root by metal transporters (Kim and Guerinot 2007). Strategy II plants acquire Fe through naturally synthesized mugineic acid (MA) family phytosiderophores, which dissolve insoluble Fe^{3+} in the rhizosphere and acquire Fe(III)-MA complexes (Marschner et al. 1986). Groundnut adopts strategy I, but is found to be inefficient for iron-use efficiency (Fageria et al. 1994).

In India, more than one-third of the soils are calcareous and spread mostly in the low rainfall areas of the western and central parts of the country, where groundnut is a major crop. Hence, IDC is more prevalent in the Saurashtra Region of Gujarat, Marathwada Region of Maharashtra, and parts of Rajasthan, Tamil Nadu, and Karnataka states in India causing considerable reduction in pod yield (16-32%) (Singh 2001; Singh et al. 1995). IDC is also a common-problem in groundnut-producing areas with calcareous soils in northern China (Li and Yan-Xi 2007) and Pakistan (Akhtar et al. 2013; Imtiaz et al. 2010) causing a significant reduction in yield. Severity of IDC will be usually quite high after excessive rainfall and also for groundnut grown under irrigation due to high bicarbonate ion concentration in the rhizosphere (Singh et al. 1995; Zuo et al. 2007).

Iron deficiency in groundnut initially appears as chlorosis on young rapidly expanding leaves which is characterized by interveinal chlorosis. During severe deficiency, veins also become chlorotic, leaves become white and papery and later turn brown and necrotic, while the plants show stunted growth resulting in reduced yield, seed Fe content, and fodder. Acute iron deficiency leads to death of plants and complete crop failure. Soil application of Fe as ferrous sulphate has often been recommended to alleviate the problem of iron chlorosis and also concomitant loss in yield (Irmak et al. 2012; Singh and Devi Dayal 1992). However, this is of little benefit to the crop as iron ionizes and gets converted into insoluble ferric (Fe^{3+}) compounds which are unavailable to plants. Foliar application of ferrous sulphate has been often suggested (Frenkel et al. 2004; Singh et al. 1993), but the major problem is poor translocation of applied Fe within the plant (Hüve et al. 2003). Although foliar spray of chelated form provides Fe in available form, their use is not popular and economically not feasible in the semi-arid tropics where groundnut is mainly grown as a rainfed subsistence crop.

IDC response is usually assessed by visual chlorosis rating (VCR) and SPAD values in groundnut (Li and Yan-Xi 2007; Samdur et al. 1999, 2000) and also other legumes like soybean, dry bean, etc. SPAD values are an indirect measurement of chlorophyll concentration based on the transmission of red light (at 650 nm) and infrared light (at 940 nm) through a leaf sample. Higher SPAD values indicate a lower incidence of leaf chlorosis. Higher VCR and lower SPAD values indicates susceptibility, while lower VCR and higher SPAD values indicates resistance to IDC.

Iron plays a double role in cell metabolism wherein, Fe is either a constituent or a cofactor of many oxidant enzymes, and can act as a pro-oxidant factor because free or loosely bound it catalyses free radical generation in the presence of reductants and peroxides through the Fenton reaction. As the intrinsic constituent or metal cofactor, Fe is actively involved in cellular detoxification reactions catalysed by catalase, phenolic-dependent peroxidases, ascorbate peroxidases, and Fe superoxide dismutase, which scavenge hydrogen peroxide and superoxide, thus protecting the cell from oxidative injury (Ranieri et al. 2001). Peroxidases (PODs) are a large family of ubiquitous enzymes which contain iron as heme and are responsible for both scavenging of H_2O_2 by the oxidation of phenols, and its generation through the oxidation of NADH. Ascorbate PODs are involved in detoxification of H_2O_2 in chloroplasts and cytosol, while 'non-specific' PODs perform different functions by catalyzing the oxidation of a wide range of phenolic substrates.

Development of micronutrient efficient genotypes can be a successive tool to overcome the micronutrient disorders in soil and also for the improvement in human health (Imtiaz et al. 2010). Genetic variability for IDC response has been reported earlier in groundnut (Su et al. 2015; Akhtar et al. 2013; Li and Yan-Xi 2007; Samdur et al. 2000;), which need to be exploited for cultivar development. Growing of IDC resistant groundnut cultivars under calcareous soils have shown significantly higher pod yield compared to susceptible cultivars (Li and Yan-Xi 2007; Prasad et al. 2000; Samdur et al. 1999). In the present study, genotypic differences were assessed for five morpho-physiological parameters associated with IDC resistance across growth stages and also yield related traits under normal- and deficit-Fe conditions.

Materials and Methods

Material & experimental design

Based on the earlier field evaluation (Boodi et al. 2015) under Fe-deficient (for Fe^{2+}) calcareous vertisols at College of Agriculture, Vijayapur, Karnataka, India (16°49" N, 75°43" E, 593 m above mean sea level), groundnut genotypes with differential IDC response [ICGV 86031, A30b (resistant); TG 26 (moderately resistant); TAG 24, TMV 2 (susceptible)] were chosen for the pot experiment. The experiment was conducted using factorial design with two major factors, i) soil Fe status ['normal-Fe' using vertisol soils with critical limit of Fe^{2+} >4.5 mg kg^{-1}; 'deficit-Fe' using calcareous vertisol soils with Fe^{2+} <4.5 mg kg^{-1}], and ii) genotypes (5) with differential IDC response. Properties of the normal- and deficit- Fe soils used for filling the pots are presented in Table 1. In total, the experiment comprised of 10 treatments

Table 1. Properties of normal-and deficit-Fe soils used for pot experimen

Soil properties	Normal-Fesoil	Deficit-Fesoil
Chemical properties		
Soil pH (1:2.5)	7.92	8.56
Electrical conductivity (1:2.5) (dS m^{-1})	0.32	0.24
Organic carbon (%)	0.54	0.63
Exchangeable calcium [c mol (P+) kg^{-1}]	15.08	22.50
Free $CaCO_3$ (%)	8.50	11.80
Available nutrients		
Nitrogen (kg ha^{-1})	388.0	328.0
Phosphorus (kg ha^{-1})	25.0	20.2
Potassium (kg ha^{-1})	550.0	510.0
Sulphur (kg ha^{-1})	15.4	14.3
DTPA extractable-Fe (mg kg^{-1})	6.72	3.76

which were grown as four replicates at College of Agriculture, Vijayapur. Five plants of each genotype were grown in cement concrete pot [18 × 18 inch, diameter and height] filled with 70 kg per pot of respective soil type (normal- or deficient- Fe). All the major nutrients (N, P, K) were supplied in the form of urea, diammonium phosphate, and muriate of potash fertilizers as per recommended dose. Micronutrients like Zn, Mn, and Mg were applied in the form of $ZnSO_4$, $MnSO_4$, and $MgSO_4$ to avoid the complexity of overlapping deficiency symptoms with Fe. Iron containing fertilizers were not applied for both normal- and deficit-Fe treatments. Management practices were followed to maintain healthy plants. Genotypic differences for morpho-physiological parameters associated with IDC resistance, yield and its related traits were assessed as described below.

Methodology

1. *Morpho-physiological parameters* – All the following observations except VCR, were recorded on standard leaf (third fully opened leaf from the top on main stem) of five plants each in every treatment to estimate mean. Such means were estimated among four replications each in normal- and deficit- Fe soils at five different stages viz., 20, 40, 60, 80, and 100 days after sowing (d). Methodology followed for recording various observations is presented below.

i) *Visual chlorosis rating (VCR)* – The 1-5 scale for VCR proposed by Singh and Chaudhari (1993) based on severity and coverage of interveinal chlorosis in entire plant was followed [1 – normal green leaves with no chlorosis, 2 – green leaves but with slight chlorosis on some leaves, 3 – moderate chlorosis on several leaves, 4 – moderate chlorosis on most of the leaves, 5 – severe chlorosis on all the leaves] (Supplementary Fig. 1). The scoring was done based on overall expression of all plants in a treatment at five different stages. The minute differences in the deficiency symptoms of different relevant micronutrients were kept in mind while scoring. Based on mean VCR score across stages, genotypes were classified as resistant (1 to 2), moderately resistant (>2 to 3), and susceptible (>3 to 5) to IDC.

ii) *SPAD values* – The SPAD values were recorded in interveinal areas of the standard leaf using the chlorophyll meter SPAD 502 (Soil Plant Analysis Development Meter, Konica Minolta).

iii) *Chlorophyll content* – The chlorophyll (a, b and total) content in the standard leaf was estimated using the method of Shoaf and Lium (1976). Fresh leaf tissue of 100 mg was cut into small pieces and incubated in 7 ml of dimethyl sulfoxide at 65°C for 30 min. At the end of incubation period, the supernatant was decanted and leaf tissue was discarded. The supernatant volume was made up to 10 ml and absorbance was recorded at 645, 652, and 663 nm in UV-Vis spectrophotometer (SL 159, ELICO, India). The total chlorophyll, chlorophyll 'a' and 'b' content were estimated using the formulae given by Arnon (1949) and expressed as mg g^{-1} on fresh weight basis.

iv) *Active Fe* – Active Fe (Fe^{2+}, ferrous) content was estimated as per the procedure of Katyal and Sharma (1980). O-phenanthroline solution required for estimation of active Fe was prepared by adding 15 g of o-phenanthroline to 850 ml of distilled water; to this continuously stirring solution, 1 N HCl was added drop-wise until the last traces of the salt were soluble and pH was around 5.5, and the final volume was made up to 1 litre. The standard leaves of plants collected were washed with tap water followed by 0.1 N HCl and then rinsed with double distilled water. Fresh leaves (2 g) were chopped with stainless steel knife and immediately transferred to glass bottles and 20 ml of o-phenanthroline solution was added and stirred gently to embathe the plant sample with the extractant. The bottles were stopped and allowed to stand for about 16 h at room temperature. The contents were filtered through Whatman No. 1 filter paper and active Fe was directly estimated in the filtrate by measuring the transmittance at 510 nm in UV-Vis spectrophotometer (SL 159, ELICO, India) and expressed as mg kg^{-1} on fresh weight basis.

v) *Peroxidase activity* – Fresh leaf tissue of 1 g was extracted with 3 ml of 0.1 M phosphate buffer (pH 6.0) by grinding with a pre-cooled mortar and pestle. The mixture was centrifuged at 3,000 rpm at 5 °C for 15 min. and the supernatant was used as enzyme source. Peroxidase activity was estimated using the method of Mahadevan and Sridhar (1986) for which, 3 ml of buffer solution, 0.05 ml guaicol solution, 0.1 ml enzyme extract, and 0.03 ml hydrogen peroxide (H_2O_2) solution were pipetted into a cuvette and mixed well and cuvette was placed in the UV-Vis spectrophotometer (SL 159, ELICO, India) at 436 nm. The change in absorbance was noted at an interval of 20 seconds for one minute after adding 0.5 ml of 2% H_2O_2 and inverting the cuvette. The protein content of enzyme extract was determined by Lowry's method (Lowry et al. 1951). The peroxidase activity was expressed as change in optical density i.e., ΔOD min^{-1} mg^{-1} of protein.

2. *Yield and its related traits* – Mean performance of genotypes for yield and its related traits like main stem height (cm) and number of primary branches per plant were recorded prior to harvest, while pod yield (g $plant^{-1}$), shelling per-

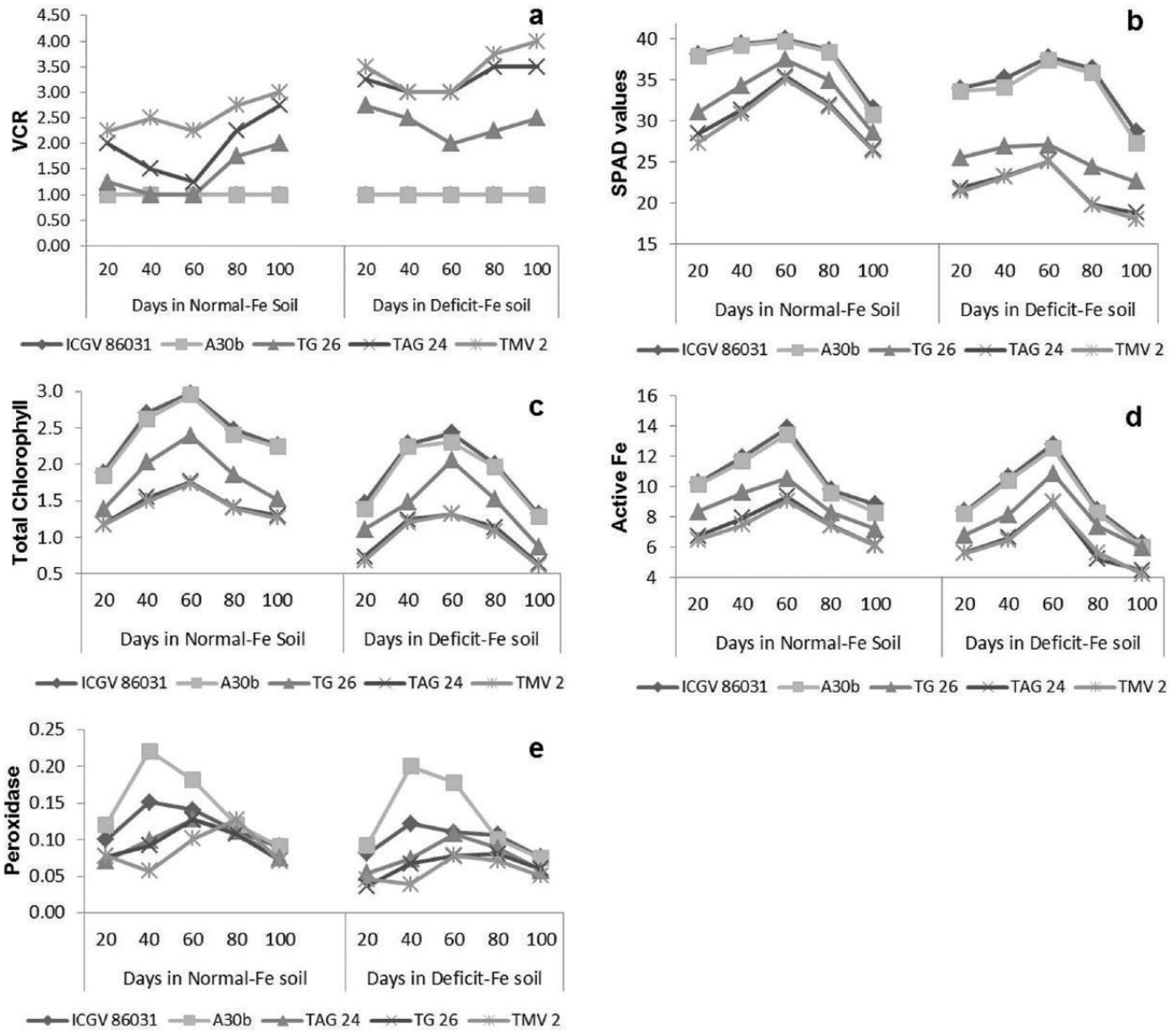

Fig. 1. Differential response of groundnut genotypes for morpho-physiological parameters under normal– and deficit– Fe soils across five growth stages: (a) Visual chlorosis rating (VCR) (b) SPAD values (c) Total chlorophyll content (mg g^{-1} fresh weight) (d) Active Fe content (mg kg^{-1} fresh weight) (e) Peroxidase activity (ΔOD min^{-1} mg^{-1} protein)

Note: ICGV 86031, A30b– Resistant; TG 26– Moderately resistant; TAG 24, TMV 2– Susceptible

centage, 100-seed weight (g), number of pods per plant, and dry haulm yield (g $plant^{-1}$) were recorded after harvest for all the treatments.

Statistical analysis

Mean squares for IDC resistance associated traits, yield and its related traits were estimated using SAS version 9.2 (SAS, Inc., Cary, NC) using generalized linear model and significance was tested using F-test. Comparison between the treatments was made by using common least significant difference ($P = 0.05$). Grouping of the genotypes within normal- and deficit-Fe conditions was done by using Tukey's HSD (honestly significant difference) test ($P = 0.05$). Pearson's correlation coefficients (r) between IDC resistance associated traits across five stages, yield and its related traits were estimated for deficit-Fe soils and significance was tested ($P = 0.01$) using table 'r' value at (n-2) degrees of freedom.

Results

Mean squares

Mean squares for morpho-physiological parameters like VCR, SPAD values, active Fe, chlorophyll (a, b, and total), and peroxidase activity at all the five growth stages (20, 40, 60, 80, and 100 days (d) after sowing) showed highly significant differences for the factors i.e., soil Fe status (A) and genotypes (B), except few cases (Supplementary Table 1).

Table 2. Mean performance of genotypes for morpho-physiological parameters in normal- and deficit- Fe soils across growth stages.

Trait*	Genotype	20 d		40 d		60 d		80 d		100 d		Mean		
		N†	D†	N	D	N	D	N	D	N	D	N	D	change(%)‡
VCR	ICGV 86031	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00	1.00	0.0
	A30b	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00	1.00	0.0
	TG 26	1.25^{a}	2.75^{b}	1.00^{a}	2.50^{b}	1.00^{a}	2.00^{b}	1.75^{b}	2.25^{b}	2.00^{b}	2.50^{b}	1.40	2.40	71.4
	TAG 24	2.00^{b}	3.25^{b}	1.50^{a}	3.00^{b}	1.25^{a}	3.00^{c}	2.25^{bc}	3.50^{c}	2.75^{c}	3.50^{c}	1.95	3.25	66.7
	TMV 2	2.25^{b}	3.50^{b}	2.50^{b}	3.00^{b}	2.25^{b}	3.00^{c}	2.75^{c}	3.75^{c}	3.00^{c}	4.00^{c}	2.55	3.45	35.3
	LSD #	0.51	0.67	0.56	0.40	0.42	0.34	0.51	0.67	0.34	0.56	-	-	-
	LSD(N&D) $	0.54		0.44		0.35		0.59		0.46		-		
SPAD	ICGV 86031	38.2^{a}	34.0^{a}	39.4^{a}	35.3^{a}	40.0^{a}	37.8^{a}	38.7^{a}	36.4^{a}	31.5^{a}	28.7^{a}	37.6	34.4	8.5
	A30b	37.9^{a}	33.6^{a}	39.3^{a}	34.1^{a}	39.8^{ab}	37.4^{a}	38.4^{a}	35.9^{a}	30.8^{a}	27.4^{a}	37.2	33.7	9.4
	TG 26	31.1^{b}	25.6^{b}	34.4^{b}	26.9^{b}	37.5^{bc}	27.1^{b}	35.0^{b}	24.5^{b}	28.6^{ab}	22.6^{b}	33.3	25.3	24.0
	TAG 24	28.5^{b}	21.8^{b}	31.4^{b}	23.3^{c}	35.4^{cd}	25.1^{b}	32.0^{c}	19.8^{c}	26.5^{b}	18.8^{bc}	30.8	21.8	29.2
	TMV 2	27.3^{b}	21.4^{b}	30.9^{b}	23.2^{c}	35.2^{d}	25.1^{b}	31.7^{c}	19.7^{c}	26.4^{b}	18.1^{c}	30.3	21.5	29.0
	LSD	3.33	2.98	3.05	1.79	1.57	2.40	1.91	2.88	2.57	3.04	-	-	-
	LSD(N&D)	3.40		2.40		1.83		2.25		2.75		-		
Active Fe	ICGV 86031	10.22^{a}	8.38^{a}	11.94^{a}	10.61^{a}	13.84^{a}	12.77^{a}	9.79^{a}	8.43^{a}	8.81^{a}	6.23^{a}	10.92	9.28	15.0
	A30b	10.10^{a}	8.22^{a}	11.65^{a}	10.39^{a}	13.42^{ab}	12.47^{a}	9.56^{a}	8.24^{a}	8.25^{a}	6.01^{a}	10.60	9.07	14.4
	TG 26	8.35^{ab}	6.78^{b}	9.60^{ab}	8.15^{b}	10.51^{bc}	10.86^{b}	8.27^{ab}	7.38^{a}	7.18^{ab}	5.96^{ab}	8.78	7.83	10.8
	TAG 24	6.74^{b}	5.63^{b}	7.92^{b}	6.64^{b}	9.33^{c}	8.98^{c}	7.43^{b}	5.22^{b}	6.10^{b}	4.46^{bc}	7.50	6.19	17.5
	TMV 2	6.46^{b}	5.59^{b}	7.47^{b}	6.47^{b}	9.04^{c}	8.95^{c}	7.41^{b}	5.62^{b}	6.09^{b}	4.22^{c}	7.29	6.17	15.4
	LSD	1.58	0.82	2.08	1.41	2.05	1.05	1.36	0.92	1.26	1.03	-	-	-
	LSD(N&D)	1.20		1.66		1.46		1.24		1.03				
Chl. a	ICGV 86031	1.26^{a}	1.00^{a}	1.66^{a}	1.48^{a}	1.83^{a}	1.58^{a}	1.57^{a}		1.13^{a}	0.87^{a}		1.26	15.4
	A30b	1.24^{a}	0.93^{ab}	1.64^{a}	1.47^{a}	1.82^{a}	1.47^{a}	1.54^{a}	1.3^{a}	1.12^{a}	0.84^{a}	1.49	1.21	17.7
	TG 26	0.94^{b}	0.74^{bc}	1.22^{b}	0.91^{b}	1.40^{b}	1.32^{b}	1.16^{b}	1.34^{a}	0.76^{b}	0.61^{b}	1.47	0.94	14.5
	TAG 24	0.83^{b}	0.55^{cd}	0.96^{c}	0.84^{b}	0.99^{c}	0.92^{c}	0.83^{c}	1.10^{b}	0.65^{b}	0.48^{b}	1.10	0.72	15.3
Chl. b	TMV 2	0.84^{b}	0.52^{d}	0.94^{c}	0.86^{b}	0.98^{c}	0.91^{c}	0.82^{c}	0.78^{c}	0.63^{b}	0.46^{b}	0.85	0.70	16.7
	LSD	0.14	0.14	0.12	0.11	0.16	0.10	0.10	0.75^{c}	0.13	0.12	0.84	-	-
	LSD(N&D)	0.14	0.48^{a}	0.11	0.80^{a}	0.12	0.85^{a}	0.12	0.14	0.12	0.45^{a}	-		
	ICGV 86031	0.63^{a}	0.45^{a}	1.04^{a}	0.77^{a}	1.14^{a}	0.83^{a}	0.91a	0.65^{a}	1.13^{a}	0.44^{a}	-	0.64	34.0
	A30b	0.60^{a}	0.38^{b}	0.99^{ab}	0.57^{b}	1.13^{a}	0.74^{a}	0.87^{a}	0.62^{a}	1.12^{a}	0.27^{b}	0.97	0.62	34.0
	TG 26	0.44^{b}	0.18^{c}	0.80^{b}	0.41^{c}	1.00^{a}	0.39^{b}	0.69^{b}	0.42^{b}	0.76^{b}	0.16^{b}	0.94	0.47	36.5
	TAG 24	0.34^{c}	0.16^{c}	0.59^{c}	0.35^{c}	0.77^{b}	0.41^{b}	0.59^{c}	0.36^{b}	0.65^{b}	0.15^{b}	.74	0.30	49.2
Total Chl.	TMV 2	0.33^{c}	0.04	0.55^{c}	0.08	0.76^{b}	0.10	0.57^{c}	0.34^{b}	0.63^{b}	0.08	0.59	0.28	50.9
	LSD	0.05		0.14		0.11		0.07	0.08	0.11		0.57	-	-
	LSD(N&D)	0.06	1.47^{a}	0.10	2.28^{a}	0.10	2.43^{a}	0.09		2.05	1.32^{a}	-		
	ICGV 86031	1.89^{a}	1.39^{a}	2.70^{a}	2.24^{a}	2.97^{a}	2.30^{a}	2.48^{a}	2.00^{a}	2.26^{a}	1.28^{a}	-	1.90	22.8
	A30b	1.84^{a}	1.11^{b}	2.63^{a}	1.48^{b}	2.95^{a}	2.06^{b}	2.41^{a}	1.96^{a}	2.24^{a}	0.87^{b}	2.46	1.83	24.1
	TG 26	1.38^{b}	0.74^{c}	2.03^{b}	1.25^{c}	2.39^{b}	1.32^{c}	1.85^{b}	1.52^{b}	1.52^{b}	0.64^{c}	2.41	1.41	23.0
	TAG 24	1.18^{c}	0.69^{c}	1.55^{c}	1.20^{c}	1.76^{c}	1.32^{c}	1.42^{c}	1.14^{c}	1.30^{b}	0.61^{c}	1.83	1.02	29.2
Peroxidase	TMV 2	1.17^{c}	0.13	1.49^{c}	0.15	1.74^{c}	0.12	1.40^{c}	1.09^{c}	1.27^{b}	0.15	1.44	0.98	30.5
	LSD	0.14		0.17		0.22		0.13	0.14	0.24		1.41	-	-
	LSD(N&D)	0.14	0.081^{a}	0.16	0.122^{b}	0.16	0.110^{b}	0.14		0.19	0.076^{a}	-		
	ICGV 86031	0.100^{ab}	0.093^{a}	0.151^{b}	0.200^{a}	0.141^{b}	0.178^{a}	0.112^{a}	0.106^{a}	0.091^{a}	0.075^{a}	-	0.099	16.8
	A30b	0.121^{a}	0.055^{b}	0.221^{a}	0.074^{c}	0.181^{a}	0.108^{bc}	0.121^{a}	0.101^{ab}	0.091^{a}	0.058^{b}	0.119	0.129	12.2
	TG 26	0.071^{c}	0.037^{b}	0.099^{c}	0.067^{cd}	0.128^{bc}	0.078^{c}	0.111^{a}	0.088^{a-c}	0.074^{a}	0.060^{ab}	0.147	0.076	21.6
	TAG 24	0.077^{bc}	0.046^{b}	0.092^{cd}	0.039^{d}	0.127^{bc}	0.079^{bc}	0.108^{a}	0.080^{bc}	0.071^{a}	0.050^{b}	0.097	0.064	32.6
	TMV 2	0.078^{bc}	0.014	0.057^{d}	0.023	0.101^{c}	0.021	0.127^{a}	0.071^{c}	0.070^{a}	0.011	0.095	0.057	34.5
	LSD	0.019		0.027		0.019		0.029	0.014	0.017		0.087	-	-
	LSD(N&D)	0.011		0.016		0.014		0.015		0.010		-		

*VCR- Visual chlorosis rating, SPAD- SPAD values, Chl. a- Chlorophyll a, Chl. b- Chlorophyll b, Total Chl.- Total Chlorophyll; d - days after sowing
#LSD- Least significant difference (*P*=0.05) for normal- and deficit-Fe soils individually; $LSD(N&D)- Common LSD (*P*=0.05) for both normal- and deficit-Fe soils for treatment comparisons; †N- Normal-Fe soil, D- Deficit-Fe soil; Initials (a, b, c, etc.) given for mean values indicate grouping of genotypes based on Tukey's HSD test within normal-Fe (N) and deficit-Fe (D) conditions;‡ Change (%)- % Change for mean across five stages between normal- and deficit-Fe i.e. % increase in VCR, while % decrease for rest other traits

Interaction (A˘B) mean squares showed significant differences for VCR at all five stages, while at specific stages for SPAD (60, 80, and 100 d), chlorophyll content ['a' (60 d), 'b' (100 d), and total (100 d)], and peroxidase activity (40 and 100 d). Highly significant differences were observed for mean squares of yield and its related traits for soil Fe status (A) and genotypes (B), except haulm yield for soil Fe status (B) (Supplementary Table 2). The interaction (A˘B) mean squares showed significant difference only for 100-seed weight.

Morpho-physiological parameters

Table 3. Mean performance of genotypes for yield and its related traits in normal- and deficit- Fe soils.

Geno type	Pod yield (g plant^{-1})			Shelling percentage			100-seed weight (g)			Main stem height (cm)			No. of primary branches per plant			No. of pods per plant			Dry haulm yield(g plant^{-1})		
	N†	D†	Redn (%)‡	N	D	Redn (%)	N	D	Redn (%)	N	D	Redn (%)	N	D	Redn (%)	N	D	Redn (%)	N	D	Redn (%)
ICGV 86031	13.6^{a}	12.7^{a}	6.6	60.9^{bc}	57.8^{b}	5.1	41.3^{a}	39.6^{a}	4.1	24.6^{a}	20.2^{a}	17.9	6.6^{a}	4.9^{a}	25.8	19.1^{a}	16.5^{a}	13.6	17.7^{a}	16.2^{a}	8.5
A30b	13.3^{a}	11.8^{a}	11.3	58.8^{cd}	56.3^{b}	4.3	39.4^{ab}	37.1^{a}	5.8	23.6^{a}	19.2^{a}	18.6	5.9^{a}	4.4^{ab}	25.4	18.3^{a}	15.2^{a}	16.9	14.0^{b}	13.5^{b}	3.6
TG 26	11.9^{a}	10.2^{ab}	14.3	62.5^{ab}	59.6^{ab}	4.6	36.3^{bc}	31.4^{b}	13.5	12.1^{bc}	11.9^{bc}	1.7	4.9^{b}	3.7^{ab}	24.5	15.7^{b}	12.2^{ab}	22.3	10.9^{c}	7.6^{d}	30.3
TAG 24	12.5^{a}	7.2^{bc}	42.4	57.0^{d}	52.1^{c}	8.6	34.0^{c}	24.9^{c}	26.8	10.4^{c}	9.4^{c}	9.6	3.9^{c}	3.2^{b}	17.9	14.2^{b}	9.1^{b}	35.9	10.6^{c}	6.1^{d}	42.5
TMV 2	11.2^{a}	7.1^{c}	36.6	65.8^{a}	62.8^{a}	4.6	33.5^{c}	24.4^{c}	27.2	17.2^{b}	15.0^{b}	12.8	3.9^{c}	3.1^{b}	20.5	14.3^{b}	8.9^{b}	37.8	15.6^{ab}	10.7^{c}	31.4
LSD #	1.66	2.06		2.26	2.63	-	2.32	1.73	-	3.78	2.14	-	0.51	0.97	-	1.31	4.07	-	2.05	1.41	-
LSD(N&D) $	2.71		-	2.29			1.93			3.20			1.11			2.75			2.84		

LSD-Least significant difference (*P=0.05*) for normal- and deficit-Fe soils individually;$ LSD(N&D)-Common LSD (*P=0.05*) for both normal- and deficit-Fe soils for treatment comparisons

†N- Normal-Fe soil, D- Deficit-Fe soil; Initials (a, b, c, etc.) given for mean values indicate grouping of genotypes based on Tukey's HSD test within normal-Fe (N) and deficit-Fe (D) conditions; ‡Redn.(%) -Reduction (%) in deficit-Fe compared to normal-Fe soil

The lowest VCR scores were recorded by resistant (ICGV 86031, A30b) followed by moderately resistant (TG 26) genotypes, while susceptible (TMV 2, TAG 24) ones showed highest VCR during all the five crop growth stages (20, 40, 60, 80, and 100 d) under deficit-Fe soils (Table 2). Comparison between normal- and deficit-Fe soils indicated that resistant genotypes did not show any change in VCR scores, while susceptible ones showed significantly higher VCR in deficit-Fe soils. Under deficit-Fe soils, VCR scores for susceptible genotypes were higher initially (20 d), further maintained or reduced at intermediate stage (40, 60 d), and increased at later stages (80, 100 d) (Fig. 1a). This shows the trend of initial susceptibility followed by slight recovery at intermediate stage, while increased susceptibility to IDC at later stages.

SPAD values were significantly higher among resistant followed by moderately resistant genotypes, but significantly lesser among susceptible ones during all the five crop growth stages in deficit-Fe soils (Table 2). Comparison between normal- and deficit-Fe soils indicated lesser reduction in SPAD values among resistant genotypes, while significant reduction among susceptible ones for SPAD across all five stages in deficit-Fe soils. Under deficit-Fe soils, SPAD values were comparatively lower during the initial stage (20 d), further increased during intermediate stage (40, 60 d), and decreased thereafter during later stage (80, 100 d) (Fig. 1b) showing the same trend of IDC response as evident from VCR scores.

Chlorophyll (a, b, and total) (mg g^{-1}), active Fe (mg kg^{-1}), and peroxidase activity (ΔOD min^{-1} mg^{-1} protein) were significantly higher among resistant followed by moderately resistant genotypes, but susceptible ones showed significantly lesser content during all the five crop growth stages under deficit-Fe soils (Table 2). Comparison between normal- and deficit-Fe soils indicated lesser reduction in chlorophyll (a, b, and total), active Fe, and peroxidase activity among resistant genotypes, while significantly higher reduction among susceptible ones across all five stages in deficit-Fe soils. Gradual increase in chlorophyll and active Fe content was observed from 20 to 60 d, but decline their onwards until 100 d was evident for all the genotypes in deficit- Fe soils (Fig. 1c, d). However, for peroxidase activity, the same trend was maintained by susceptible genotypes, but resistant types showed increase up to 40 d and decrease their onwards from 60 to 100 d (Fig. 1e).

Table 4. Associations betweenmorpho-physiological parameters considering all five stages in deficit-Fe soils.

Trait[a]	SPAD	Active Fe	Chl.a	Chl.b	Tot. chl.	Peroxidase
VCR	-0.923**	-0.656**	-0.768**	-0.775**	-0.776**	-0.654**
SPAD		0.809**	0.869**	0.861**	0.872**	0.719**
Active Fe			0.903**	0.913**	0.913**	0.768**
Chl.a				0.971**	0.996**	0.828**
Chl.b					0.989**	0.832**
Tot. chl.						0.835**

[a]VCR- Visual chlorosis rating, SPAD-SPAD values, Chl. a-Chlorophyll a, Chl. b- Chlorophyll b, Total Chl.-Total Chlorophyll; ** Significant difference at *P*=0.01

Under deficit-Fe conditions, for most of the traits at different stages based on Tukey's HSD test indicated distinct grouping of resistant (ICGV 86031, A30b) and susceptible (TAG 24, TMV 2) genotypes, while moderately resistant genotype (TG 26) in majority of cases grouped with susceptible ones (Table 2). Comparison between normal- and deficit-Fe soils for mean across five stages indicated: i) least reduction in IDC resistant genotypes compared to susceptible ones for SPAD (8.5-9.4%, 29.0-29.2%), chlorophyll 'b' (34%, 49.2-50.9%), total chlorophyll (22.8-24.1%, 29.2-30.5%), and peroxidase activity (12.2-16.8%, 32.6-34.5%), respectively; ii) Among resistant and susceptible genotypes, least differences were observed for active Fe (14.4-15.0%, 15.4-17.5%), while almost no change for chlorophyll 'a' (15.4-17.7%, 15.3-16.7%), respectively (Table 2).

Yield-related traits

IDC resistant genotypes were found superior for yield and its related traits compared to moderately resistant and susceptible genotypes under deficit-Fe conditions (Table 3). Under

Table 5. Associations between mean of morpho-physiological parameters across five stages and yield-related traits

Traita	Pod yield	Shelling percentage	100-seed weight	Main stem height	No. of primary branches	No. of pods	Dry haulm yield
VCR	-0.978**	0.130	-0.988**	-0.815	-0.976**	-0.988**	-0.787
SPAD	0.987**	-0.105	0.982**	0.864	0.985**	0.985**	0.846
Active Fe	0.972**	-0.035	0.995**	0.795	0.971**	0.993**	0.767
Chl. a	0.987**	-0.075	0.997**	0.828	0.986**	0.997**	0.807
Chl. b	0.974**	-0.070	0.996**	0.784	0.972**	0.994**	0.757
Tot. chl.	0.983**	-0.078	0.997**	0.812	0.981**	0.996**	0.788
Peroxidase	0.836	-0.222	0.861	0.738	0.831	0.859	0.664

[a]VCR- Visual chlorosis rating, SPAD- SPAD values, Chl. a- Chlorophyll a, Chl. b- Chlorophyll b, Total Chl.- Total Chlorophyll; ** Significant difference at *P*=0.01

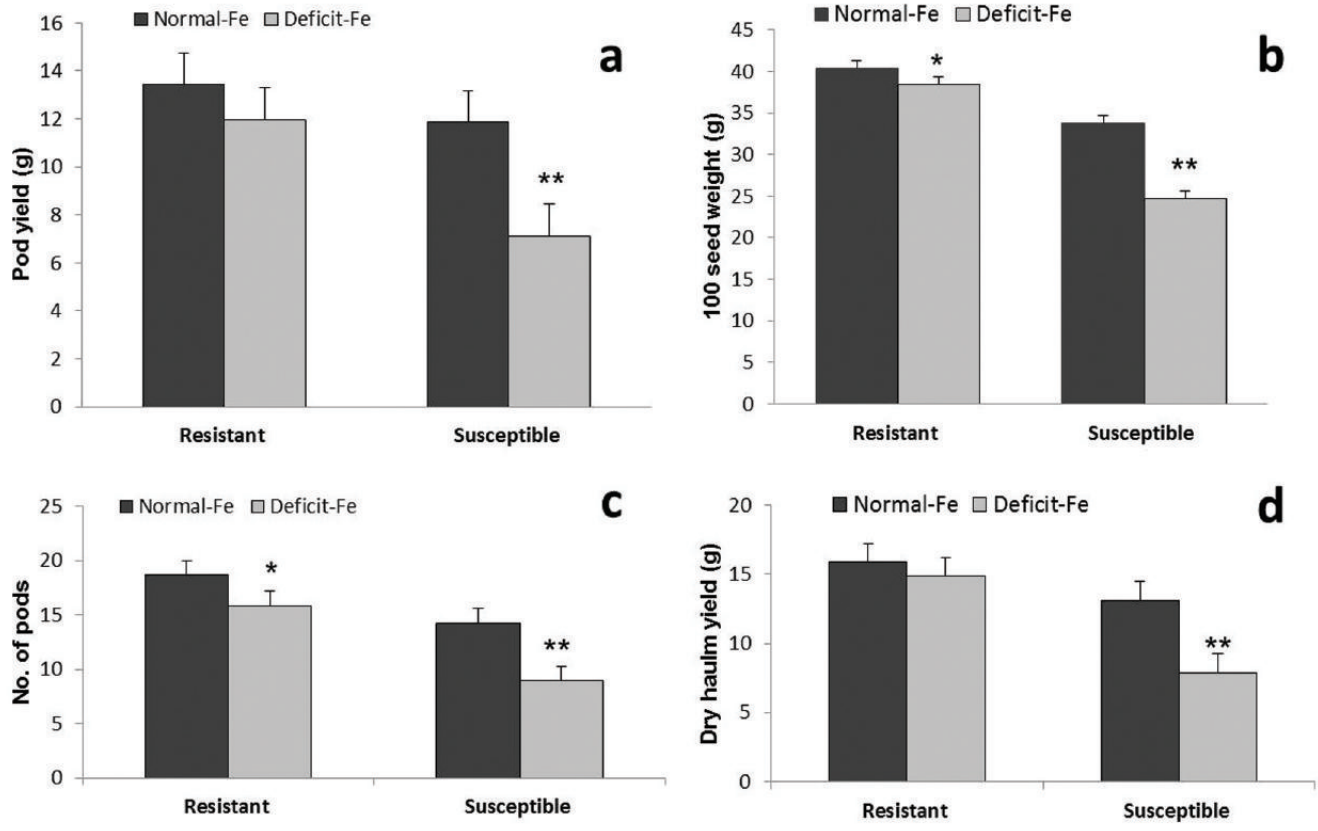

Fig. 2. Differences among IDC resistant and susceptible groundnut genotypes under normal– and deficit–Fe soils for yield and its related traits (a) Pod yield (g plant^{-1}) (b) 100-seed weight (g) (c) Number of pods per plant (d) Dry haulm yield (g plant^{-1})
Note: Mean of Resistant (ICGV 86031, A30b) and Susceptible (TAG 24, TMV 2) genotypes considered for comparison; Standard error bar is common for both resistant and susceptible genotypes
*,** Significant difference between normal– and deficit–Fe soil at $P = 0.05$ and 0.01, respectively

deficit-Fe conditions, for majority of the yield trait based on Tukey's HSD test indicated distinct grouping of resistant (ICGV 86031, A30b) genotypes from susceptible (TAG 24, TMV 2) ones, while moderately resistant genotype (TG 26) either grouped with susceptible or resistant genotypes (Table 3). Comparison between normal- and deficit-Fe soils indicated least reduction in resistant genotypes compared to susceptible ones for pod yield (6.6-11.3%, 36.6-42.4%), 100-seed weight (4.1-5.8%, 26.8-27.2%), dry haulm yield (3.6-8.5%, 31.4-42.5%), and number of pods (13.6-16.9%, 35.9-37.8%), respectively (Table 3, Fig. 2). Contrastingly, more reduction in resistant genotypes compared to susceptible ones was observed for main stem height (17.9-18.6%, 9.6-12.8%) and number of primaries (25.4-25.8%, 17.9-20.5%) (Table 3, Fig. 3).

Associations

In deficit-Fe soils, highly significant ($P < 0.001$) negative correlation was observed between VCR and SPAD consider-

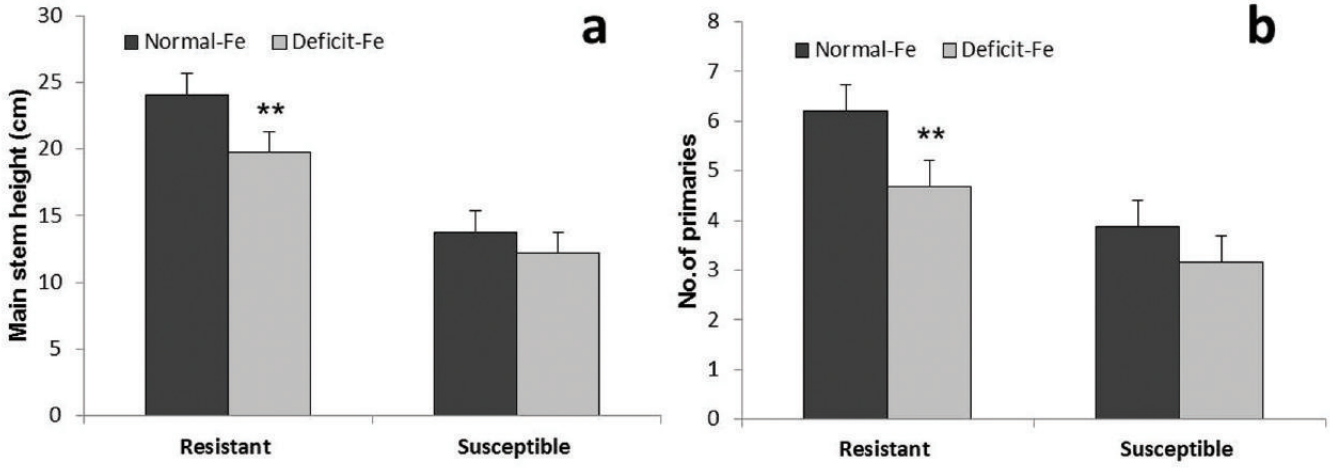

Fig. 3. Differences among IDC resistant and susceptible groundnut genotypes under normal– and deficit–Fe soils for (a) Main stem height (cm) (b) Number of primaries per plant

Note: Mean of Resistant (ICGV 86031, A30b) and Susceptible (TAG 24, TMV 2) genotypes considered for comparison; Standard error bar is common for both resistant and susceptible genotypes

** Significant difference between normal- and deficit-Fe soil at $P = 0.01$

ing all five stages (-0.923) (Table 4). VCR showed highly significant ($P < 0.01$) negative correlation, while SPAD showed highly significant ($P < 0.01$) positive correlation with parameters like active Fe, chlorophyll (a, b, and total), and peroxidase activity. VCR showed highly significant ($P < 0.01$) negative correlation, while SPAD, active Fe, and chlorophyll (a, b, and total) showed highly significant ($P < 0.01$) positive correlation with yield-related traits like pod yield per plant, 100-seed weight, number of primaries per plant, and number of pods per plant (Table 5). Pod yield showed highly significant ($P < 0.01$) positive correlation with 100-seed weight (0.989), number of primaries (1.000), and pods per plant (0.993) (data not shown). This shows their contribution to pod yield under deficit-Fe soils.

Discussion

Iron deficiency chlorosis is common in groundnut growing areas with calcareous and alkaline soils and causes moderate to severe reduction in yield (Singh 2001; Singh et al. 1995). Cultivation of IDC resistant cultivars is an economically viable option as they perform well under Fe-deficient conditions (Prasad et al. 2000; Samdur et al. 1999). To identify and utilize IDC resistance sources, one needs to identify suitable surrogate traits that are simple, robust, and reliable across crop growth stages for their effective utilization in breeding programme. Hence, an effort was made in the present study to identify the suitable surrogate trait/s by studying the behaviour of groundnut genotypes with differential IDC response across different stages for five morpho-physiological parameters associated with IDC resistance and also their impact on yield related traits.

Under deficit-Fe soils, resistant genotypes recorded very less incidence of IDC evident from low VCR and high SPAD values compared to susceptible genotypes across all five stages (20 to 100 days). This study has clearly established the utility of VCR and SPAD in assessing IDC resistance by studying across five different stages. Hence, VCR and SPAD values are extremely useful for preliminary and large-scale screening of germplasm/breeding material due to their simplicity and robustness. Wide variation for VCR and SPAD values among groundnut germplasm has been noted by earlier workers (Li and Yan-Xi 2007; Samdur et al. 1999, 2000), but detailed studies across several growth stages were not made that has been done in the present study. The progression of IDC response among susceptible genotypes as assessed by VCR and SPAD indicated their susceptibility at initial stage, while slight recovery at intermediate stage, and further becoming still more susceptible at later stages (Table 2, Figs. 1a, b). In groundnut, self-recovery of chlorosis as leaves become older has been noted, but the newly emerging leaves still show chlorosis (Singh 1994a). In soybean, Naeve and Rehm (2006) also reported that iron deficiency is often most severe early in the growing season and gradually disappears in the mid-growing season that is in line with results of present study. Earlier reports in groundnut suggest beginning of iron deficiency at 10-15 days after emergence, while attaining of maximum intensity at 30-70 days (Singh and Chaudhari 1993) or 50-65 days after emergence (Li et al. 2009a). In our study, maximum severity was observed at later stages probably because we considered days after sowing for recording observations, while earlier workers considered days after emergence making a difference of at least a week. Severity at later stages could also be due to exhaustion and limited iron-availability under pot condition.

SPAD values were able to clearly differentiate the severity

of chlorosis compared to VCR scores among genotypes evident from being lower at initial stage, increasing at intermediate stages, and getting reduced at later stages (Fig. 1b). Being a quantitative measure, SPAD is more reliable in judging IDC resistance compared to VCR that has inbuilt tendency of committing error by the scorer. SPAD value has been earlier indicated as a feasible screening indicator to select iron-resistant groundnut cultivars (Li et al. 2009b). However, the present study conclusively proves the earlier results by studying across different stages among groundnut genotypes with differential IDC response. SPAD being simple and robust, it would be ideal for large-scale screening to identify resistant sources and also breeding for IDC resistance as one can phenotype large populations.

Biochemical parameters like chlorophyll, active Fe, and peroxidase activity were assessed across five different stages to know their relevance as traits for judging IDC response. Reduced chlorophyll content in the leaves due to iron deficiency is a direct indication of interveinal chlorosis, which may or may not get noticed through visual scoring for chlorosis. Reduced chlorophyll content in leaves is at least in part due to the role of Fe in the formation of precursors of the chlorophyll molecule i.e., δ-aminolevulinic acid and protochlorophyllide (Marschner 1986). From normal- to deficit-Fe soil, much lesser reduction of chlorophyll ('b' and total) among resistant genotypes compared to susceptible ones (Table 2, Fig. 1c) is indicative of the feasibility of chlorophyll content as an indicator. Reduced chlorophyll content among IDC susceptible groundnut genotypes has been noted earlier in field experiment (Samdur et al. 2000) and hydroponics (Xiao-Ping et al. 2010). However, our study conclusively proves the relevance of chlorophyll content in judging IDC response across stages in groundnut genotypes with differential responses. SPAD is an indirect indicator of chlorophyll content as significant correlations have been established across different stages in the present study as well as by other researchers (Samdur et al. 2000). Hence, SPAD is more robust and suitable than estimation of chlorophyll content for large-scale screening of groundnut genotypes for IDC response.

Active Fe content in deficit-Fe soils was found to be higher among resistant followed by moderately resistant genotypes, while it was the lowest in susceptible ones (Fig. 1d) indicating its feasibility as an indicator for IDC resistance. Chlorotic plants have been earlier found to show lower active Fe content in leaves (Singh 1994b). Significant positive correlation between active Fe and chlorophyll (a, b, and total) content was observed in this study across different stages as noted earlier in groundnut (Akhtar et al. 2013). There was a comparatively lesser reduction in active Fe from normal- to deficit- Fe soil among resistant compared to susceptible genotypes confirming its direct role in IDC resistance as Fe is required for chlorophyll formation and photosynthesis (Zheng 2010). Active Fe content has been shown earlier to be an important indicator of IDC resistance among groundnut genotypes (Li et al. 2009b; Li and Yan-Xi 2007; Reddy et al. 1993; Singh 1994b). However, this study clearly established it by studying across stages among groundnut genotypes with differential IDC response. Since estimation of active Fe in laboratory is tedious, it could rather be used for confirmation of IDC resistance among preliminarily selected lines/ plants based on SPAD values.

Higher peroxidase activity observed among IDC resistant genotypes across all stages (Fig. 1e) has also been noted earlier in groundnut (Xiao-Ping et al. 2010). In *Medicago ciliaris*, the IDC tolerant line was found to accumulate lower levels of H_2O_2 and was better protected against oxidative damage caused by Fe starvation (M'sehli et al. 2014). Reduction in peroxidase activity was observed among all genotypes in deficit-Fe soil compared to normal-Fe soil. However, a lower reduction was observed among resistant genotypes compared to susceptible ones probably due to comparatively higher active-Fe maintained in leaves under Fe-stress conditions. Iron deficiency has been found to reduce the activity of oxidative stress-related enzymes like catalase, ascorbate peroxidase, and peroxidase in several plant species that is attributed to less Fe concentration in Fe-deficient leaves (M'sehli et al. 2014; Salama et al. 2009; Mohamed and Aly 2004; Zaharieva and Abadía 2003; Ranieri et al. 2001). Alleviation of iron deficiency stress in groundnut through application of nitric oxide and salicylic acid has also been shown to increase the peroxidase activity (Kong et al. 2014, 2015) confirming its role under IDC stress. The present results emphasize the utility of peroxidase activity as an indicator to identify IDC-resistant groundnut genotypes.

From normal- to deficit-Fe conditions, IDC led to a severe reduction in pod yield among susceptible compared to resistant genotypes (Fig. 2a). Moderate to severe yield reductions among susceptible genotypes due to IDC have been reported in groundnut (Singh 2001; Singh et al. 1995). IDC-tolerant groundnut genotypes have been found to show s higher pod yield, haulm yield, and number of pods in this study as noted earlier by Akhtar et al. (2013). The study also revealed that a lower reductions in pod yield among resistant types is mainly due to lower reduction in 100-seed weight, number of pods, and haulm yield (Figs. 2b, c, d). In contrast, a greater reduction in resistant genotypes compared to susceptible ones for main stem height and number of primaries (Fig. 3) is the first report of such kind in groundnut. This suggests that resistant genotypes manifest better yield by controlling overgrowth under iron-deficient conditions. In soybean, iron efficient plants have been generally found to be shortest (Elmstrom and Howard 1969) and a negative correlation has been noticed between stem length and IDC tolerance (Vasconcelos and Grusak 2014). Such a relation has been noted for the first time in groundnut through this study. Hence, parameters like 100-seed weight, number of pods, haulm yield, main stem height, and number of primaries could be used as a selection criterion while selecting for higher productivity under Fe-

deficient environments.

In deficit-Fe soils, physiological parameters (active Fe, chlorophyll, and peroxidase activity) showing highly significant negative correlation with VCR, while positive correlation with SPAD are suggestive of their utility for evaluation, identification, and development of IDC-resistant genotypes. VCR showing highly significant negative correlation, while morpho-physiological parameters (SPAD, active Fe, chlorophyll, and peroxidase) showing highly significant positive correlation with yield related traits are suggestive of their utility in the development of high-yielding varieties with IDC resistance. Highly significant positive correlation of pod yield with component traits like 100-seed weight, number of primaries, and pods per plant suggests their contribution in increasing pod yield under Fe-deficient conditions. Earlier reports in groundnut also suggested a significant positive correlation of pod yield with SPAD values, active Fe, number of pods and seeds (Akhtar et al. 2013; Li and Yan-Xi 2007).

Conclusions

Under deficit-Fe conditions, IDC-resistant genotypes recorded a significantly lower VCR, while higher SPAD, active Fe, chlorophyll, and peroxidase activity in leaves across all five crop growth stages compared to susceptible ones confirming their utility as traits for identification and development of IDC-resistant groundnut genotypes. However, the combined use of SPAD and active Fe is most feasible due to their simplicity, robustness, and reliability compared to other physiological parameters as correlations have been already established in this study across stages. Towards developing high-yielding, IDC-resistant groundnut cultivars for Fe-deficient environments, selection should be practised for higher 100-seed weight, number of pods, and haulm yield, while limited main stem height and number of primary branches.

Acknowledgements

This research work was done as part of post-graduation research of first author and also as part of staff research project granted to second author by the Directorate of Research, University of Agricultural Sciences, Dharwad 580005 (India).

Conflict of interest: The authors declare that they have no conflict of interest.

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