Chapter 19
In Vitro Cell Selection Techniques for Enhancing Disease Resistance – Case Study: Common Scab Resistance in Russet Burbank

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Abstract Here we demonstrate the value of *in vitro* cell selection techniques for potato cultivar improvement using enhancement of common scab resistance as a case study. Common scab is an important disease of potato world-wide. A diversity of *Streptomyces* species are associated with disease, however all pathogenic species and strains produce the phytotoxin, thaxtomin A. This toxin is an essential pathogenicity factor and provides a convenient positive selection agent for *in vitro* cell selection to obtain disease resistant potato variants. Using such techniques we obtained many variants of Russet Burbank with enhanced resistance to common scab, interestingly some of which did not express toxin tolerance. Agronomic assessments showed many variants had impaired yield, however several variants had equivalent or even superior yield to the unselected parent cultivar and these have been progressed toward commercial exploitation. Associated studies showed many of the common scab resistant variants showed resistance to a second unrelated disease, powdery scab. Preliminary studies suggest altered suberisation within lenticels may be a possible mechanism for broad spectrum disease resistance to tuber-invading pathogens.

19.1 *In Vitro* Cell Selection Versus Traditional Breeding Approaches

Management of soil-borne diseases of potato is often difficult and not particularly effective. There are limited management tools available. Fungicides or other agrichemical options may be ineffective due to the difficulty in maintaining sufficient concentrations of the active materials in the soil during the time of pathogen invasion (often many weeks after planting).

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Host plant resistance is a valuable tool to combat debilitating diseases. Most potato breeding programs around the world strive to enhance resistance to locally important soil-borne diseases, however, selecting effective disease resistance traits whilst maintaining other essential agronomic and quality features is often extremely difficult. This is particularly problematic in French fry production as strict demands are placed on tuber quality and cooking characteristics as well as yield.

An alternate approach to conventional breeding to obtain genetic disease resistance (or other traits of value) is through the use of *in vitro* cell selection to produce somaclonal variants (Shepard et al. 1980; Larkin and Scowcroft 1981; van Harten 1998). Potato is readily amenable to tissue culture and may be regenerated from undifferentiated callus with relative ease (Shepard and Totten 1977). Variants of a parent potato cultivar may be generated during tissue cell culture with or without the use of chemical mutagens. They can then be screened for specific traits of interest. Selection of somaclonal variants with traits such as enhanced disease resistance has a significant advantage over traditional breeding in that it avoids the major genetic re-assortment associated with sexual crossing. Thus the likelihood that derived variants retain most or all desirable traits of the parent cultivars is greater (Shepard et al. 1980; Larkin and Scowcroft 1981). Furthermore, the process for selection of elite cultivars is generally faster and cheaper than conventional breeding approaches.

In vitro cell selection procedures have been used to obtain or refine traits of interest in potato. These include enhanced disease resistance to pathogens such as *Phytopthora infestans, Verticillium dahliae, Alternaria solani, Fusarium oxysporum* and *Streptomyces scabiei* (Matern et al. 1978; Behnke 1980; Gunn et al. 1985; Taylor et al. 1993; Sebastiani et al. 1994).

19.2 Case Study: Selection of Resistance to Common Scab Disease in Commercial Potato Cultivars

Common scab is regarded as one of the most economically important diseases of potato in Tasmania reflecting its prevalence and disease severity. Common scab is induced following infection of developing tubers with pathogenic *Streptomyces* spp. Yield is seldom affected, but reduction in tuber quality and consequent value of fresh produce, and losses to seed tuber producers through failure of certification standards can be substantial. Deep-pitted lesions frequently occur in common scab affected tubers in Australia (Wilson et al. 1999). These affect processing quality where the normal steam-peeling processes do not remove the lesions adding substantial costs to the processing sector.

Recent industry figures estimate common scab costs the Tasmanian French fry processing industry A$3.7 M per annum (p.a.) or approximately 4% of the annual industry value (P. Hardman, Simplot Australia, personal communication). Seed producers face the largest losses. Crops may be rejected if infection levels exceed the national certification guidelines of ≤4% tubers possessing lesions. To cope with

anticipated loss of certified seed, the processing companies routinely contract 20% more seed than required. The cost to the seed sector is estimated at A$2.3 M p.a. (31% of this sector's value). Growers of processing crops face loss of tuber quality and of tuber yield (as severely affected crops are harvested early) resulting in an estimated A$0.4 M p.a loss. In addition, rejection of crops at the factory (an average of 1% of crops are rejected) due to unacceptable levels of common scab disease result in an additional A$1.0 M p.a. loss. In the factory, sophisticated processing lines can eliminate much of the lesioned tissues that escape the peeling process. Infected potatoes also will be processed according to the quality standards of the customer (the worst affected lines going to cheaper generic brand products). Reject fries and post-peel potato waste (including some lesioned materials) may be processed into hash browns or potato flour.

Management options for the control of common scab in potato are limited and generally not very effective (Loria et al. 2006). Host plant resistance is regarded as an important control strategy for durable disease management. No commercial potato cultivars are immune to common scab disease, but there is considerable variation in cultivar susceptibility (McKee 1958). Following infection the potato tuber is stimulated to produce suberised cork layers at the site of attack that limit further penetration by the pathogen (Fischl 1990). However, it is these defensive cork layers which disfigure the tubers.

Potato breeding programs around the world include enhancing resistance to common scab as one of their key priorities. This objective, however, has proved very difficult to achieve, due at least partially to the polygenic nature of known heritable resistance (Haynes et al. 1997). Furthermore, there are logistical difficulties in accurately and efficiently screening for common scab resistance within the population of seedlings from breeding crosses. Also, as mentioned above, the French fry processing industry has very strict requirements for acceptable yield, tuber size, shape, reducing sugars, solids etc. which severely limits suitable varieties.

Russet Burbank is one of the most widely grown potato cultivars in the world (comprising *c.* 40% of all potatoes grown in the USA and Australia) primarily because it possesses tuber characteristics and cooking qualities that make it ideal for French fries. Russet Burbank was first selected over 130 years ago and despite the concerted efforts of breeding programs to obtain alternative varieties with equivalent or better traits it remains the dominant cultivar for French fry production around the world. The variety is male sterile which reduces its value as a parent in conventional breeding and has meant incorporation of its genetic qualities into new varieties has not been widely attempted.

Russet Burbank possesses moderate resistance to common scab disease (Lambert et al. 2006), however, it frequently suffers severe disease under conducive conditions (Sparrow and Salardini 1997; Wilson et al. 1999; Wanner and Haynes 2009). There are several different clones of Russet Burbank characterised (Coleman et al. 2003). Small but significant variability in the levels of resistance to common scab (incidence and severity) have been shown (Wilson 2001).

19.3 Pathogen Characterisation from Tasmanian Soils

S. scabiei is the predominant species associated with this disease, although a diversity of distinct pathogenic *Streptomyces* species and strains have been identified in various studies around the world (Tashiro et al. 1990; Doering-Saad et al. 1992; Faucher et al. 1992; Bouchek-Mechiche et al. 2000; Park et al. 2003). A common feature of all pathogenic strains and species of *Streptomyces* is their ability to synthesise the thaxtomin group of phytotoxins (of which thaxtomin A is the predominant compound), whilst non-pathogenic strains fail to produce the phytotoxins (King et al. 1989, 1991; Babcock et al. 1993; Loria et al. 2006).

A selection of pathogenic isolates obtained from diseased Tasmanian potatoes have been analysed for morphological and biochemical traits (Kurster 1972), partial 16s cDNA sequencing, thaxtomin A production by thin layer chromatography, and the presence of the *nec1* gene (commonly linked with thaxtomin synthesis genes) using a PCR assay with nested primers (Cullen et al. 2000).

Examination of the 16s data showed most of these isolates fell within a single clade clustering with sequences from *S. scabiei* isolates from various parts of the world (Fig. 19.1). Morphological characteristics of these isolates varied to some extent from those of the type strain of *S scabiei* (ATCC 49173) including greater tolerance of acid growth conditions (down to pH 4.0). One diverse isolate was identified. It clustered most closely to *S. turgidiscabies* (ATCC 700248, AB026221) of the known pathogenic species (Fig. 19.1), but again possesses characters which differ from the type strain of this species. *S. turgidiscabies* was described from Japan, and has also been reported associated with common scab in Korea (Park et al. 2003) and other parts of the world. This Tasmanian isolate did not produce a melanin pigment, favoured acid growth conditions (down to pH 3.5), did not possess the *nec1* gene but did produce thaxtomin A. It is interesting to note the presence of an acid-tolerant strain from Tasmanian soils, as the soil environment is only mildly acidic and well buffered, not necessarily suggestive of a major evolutionary advantage toward acid tolerance.

19.4 *In Vitro* Selection of Disease Resistant Russet Burbank Somaclones

Thaxtomin A is thought to inhibit cellulose biosynthesis and to trigger a programmed cell death response in plants (Fry and Loria 2002; Duval et al. 2005; Bischoff et al. 2009). Experimental evidence has confirmed thaxtomin A has an essential role in the induction of common scab (Goyer et al. 1998; Healy et al. 2000; Kers et al. 2005). This requirement of thaxtomin A for disease induction has provided a specific target for resistance studies. Selecting for tolerance to thaxtomin A may be an effective way of obtaining or identifying resistance. Indeed the use of purified phytotoxin to rapidly screen new lines generated in breeding programs for

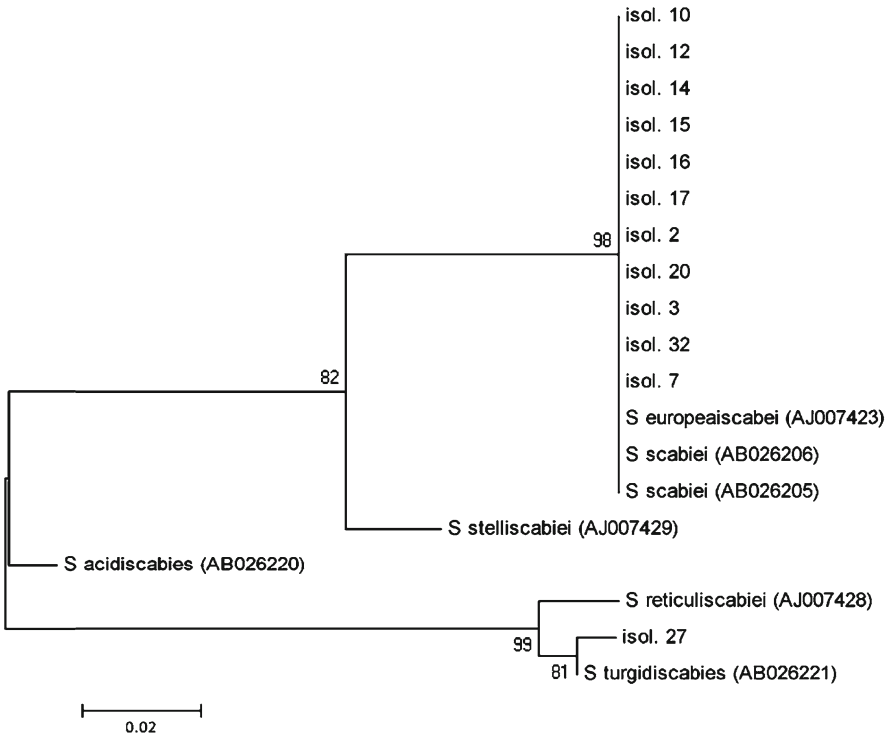

Fig. 19.1 Phylogenetic analysis of partial 16s cDNA sequences for 12 Tasmanian pathogenic *Streptomyces* isolates (compared to typed pathogenic species from GENBANK). The maximum likelihood tree was constructed using MEGA4 with 1,000 bootstrap replicates and the pairwise deletion option. Bootstrap values greater than 50% are shown

probable common scab resistance has been evaluated (Hiltunen et al. 2006). The phytotoxin also provides a convenient selection tool for *in vitro* cell selection studies to obtain toxin tolerant (and disease resistant) variants of existing commercial cultivars.

We utilised tissue culture technologies to select for thaxtomin A tolerant potato clones of current commercial varieties (Wilson et al. 2009, 2010b). This has the advantage of seeking enhanced resistance phenotypes whilst retaining important commercial characteristics of the parent varieties. The approach used thaxtomin A as a cell selection agent against cultured potato cells (callus). Incorporation of thaxtomin A into the culture medium was used to impose a toxic stress on a large population of plant cells from which rare variants tolerating the toxin treatment were recovered.

The procedure we used for *in vitro* cell selection of disease resistant potato variants is outlined in Fig. 19.2. In brief, we obtained highly purified thaxtomin A by solvent extraction and column chromatography from pathogen cultures in liquid medium (Wilson et al. 2009). Potato callus cultures were initiated from stem

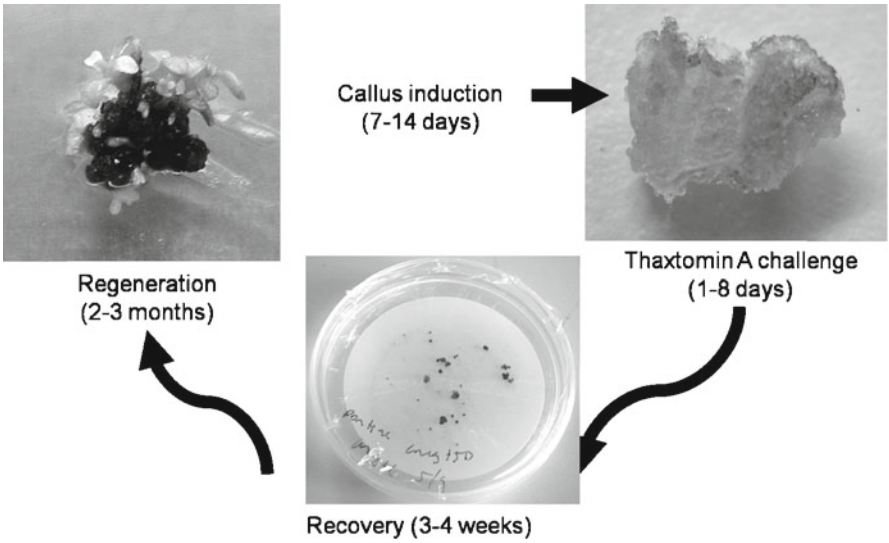

Fig. 19.2 Schematic of the *in vitro* cell selection procedure used to obtain common scab resistant potato variants

internodes of tissue culture plants of Russet Burbank. Friable callus cells were isolated from calloused stems and suspended in liquid medium containing toxic quantities of thaxtomin A (4–7 μM) and incubated for 1–8 days.

Recovery of the rare surviving potato cell colonies required amelioration of the inhibitory effect of the majority of dead and decaying cells. This was achieved by plating toxin-treated cells onto a sterile filter paper placed on top of a nurse culture of tobacco cells (*Nicotiana plumbaginifolia)* grown on a standard callus inducing medium (Conner and Meredith 1985). This was further aided by incorporation of a coconut water additive within the medium (Wilson et al. 2009, 2010b).

After 3–4 weeks, surviving potato cell colonies were identifiable. Following transferral to a regeneration medium shoots were obtained after a further 2–3 months, multiplied in tissue culture and used for subsequent assays. From 29 toxin-challenge selection trials, a total of 253 variants (from 212 cell colonies) of Russet Burbank were obtained.

19.5 Assessment for Toxin Tolerance and Disease Resistance

Variants obtained following selection were screened for thaxtomin A tolerance in comparison to the unselected parent using both detached leaflet and tuber slice bioassays (Wilson et al. 2010b). In these bioassays potato tissues were incubated with lethal concentrations (7 μM) of thaxtomin A and resulting necrosis scored.

In the leaflet bioassay necrosis scores following thaxtomin A treatment of the variants tested ranged from only 10% up to 250% of the necrosis shown by the parent with a mean result equivalent to the parent. The tuber slice bioassay gave similar although more conservative results. Between 16% and 22% of all variants tested showed enhanced thaxtomin A tolerance (with 5% showing a surprising increased sensitivity to the toxin in the leaflet bioassay; Table 19.1). Variants were consistent in their response to toxin treatment either showing enhanced thaxtomin A tolerance (e.g. A260) or no apparent change in thaxtomin A sensitivity (e.g. A380; TC-RB8; NZ-22c; Fig. 19.3).

Variants along with the unselected parent cultivar were then screened for common scab resistance in glasshouse trials. Those lines showing significantly less disease than the unselected parent cultivar were repeatedly screened in further glasshouse and subsequent field trials. Only those lines consistently showing reduced disease across all trials were considered as possessing enhanced disease resistance. In glasshouse trials inoculum (vermiculite colonised with known strains of pathogenic *S. scabiei*) was introduced to the soil medium at planting, whereas field assessment relied on natural soil inoculum. Growth conditions were adjusted to favour disease (limited irrigation and addition of lime at 1 kg/ha equivalent to the potting media or field soils). Harvested tubers were scored for disease incidence (measured by determining the proportion of tubers with visible lesions), and severity (measured by two assessments. First the tuber surface cover score was estimated visually, and second, where lesions were present on a tuber, the depth of the deepest lesion was scored; Wilson et al. 1999).

A total of 18–20% of Russet Burbank variants consistently demonstrated enhanced resistance to common scab in both incidence and severity assessments (Table 19.1) under varying disease pressure in both glasshouse and field assessment. Pooling data for all variants tested showed a mean disease reduction of 68% (incidence) and 65% (severity – tuber surface area covered with lesions) compared to the parent cultivar. The most disease resistant variants (e.g. A260 and NZ-22c) had a mean reduction in disease incidence of 97–100% and in disease severity of 98–100% (Table 19.2; Fig. 19.3). Visual symptoms were rare in the most resistant variants, while tubers of the unselected parent frequently had severe disease symptoms (Fig. 19.4).

19.6 Agronomic Assessment of Yield and Tuber Quality

In selection of somaclonal variants for valuable characteristics such as disease resistance, undesirable traits may be co-selected. These may be a direct result of the genetic change to achieve the desirable trait, or more commonly, the result of additional independent genetic changes (van Harten 1998). In order to determine the commercial merit of the disease resistant variants we undertook agronomic assessment of plant growth, yield and tuber quality (Wilson et al. 2010a).

Variants that expressed reduced mean common scab severity in one or more trials (70% or less than expressed by the parent cultivar) and four susceptible clones were

Table 19.1 Summary of toxin tolerance, disease resistance, agronomic performance and tuber quality for Russet Burbank somaclonal variants obtained by *in vitro* cell selection relative to the unselected parent cultivar

		Mean variant score relative to parent (%)	Range of variant scores relative to parent (%)	Variants significantly greater than parent (%)	Variants significantly less than parent (%)
Thaxtomin A tolerance	Leaflet bioassay	100	10–250	5	16
	Tuber slice bioassay	90	50–110	0	22
Disease resistance	Incidence (tubers with lesions)	32	0–156	0	20
	Severity (lesion surface coverage)	35	0–176	0	20
	Severity (lesion depth)	90	38–253	0	18
Tuber yield	Tuber number	36	2–121	0	9
	Tuber weight (total)	66	2–124	3	68
	Tuber weight (fry grade)	64	2–127	3	72
Tuber quality	Specific gravity	100	99–100	2	8
	Dry matter content	94	87–107	2	8
	Flesh colour	115	100–200	6	0
Cooking quality	Dark end (defect)	69	0–420	2	0
	Best fry colour (c000)	86	0–320	3	0
	Unacceptable fry colour (c1–2)	82	0–1,350	6	0
Tuber defects	Misshapen tubers	104	0–515	3	0
	Cracked tubers	57	0–790	5	0
	Hollow heart	61	0–820	2	0
	Internal browning	55	0–350	2	0

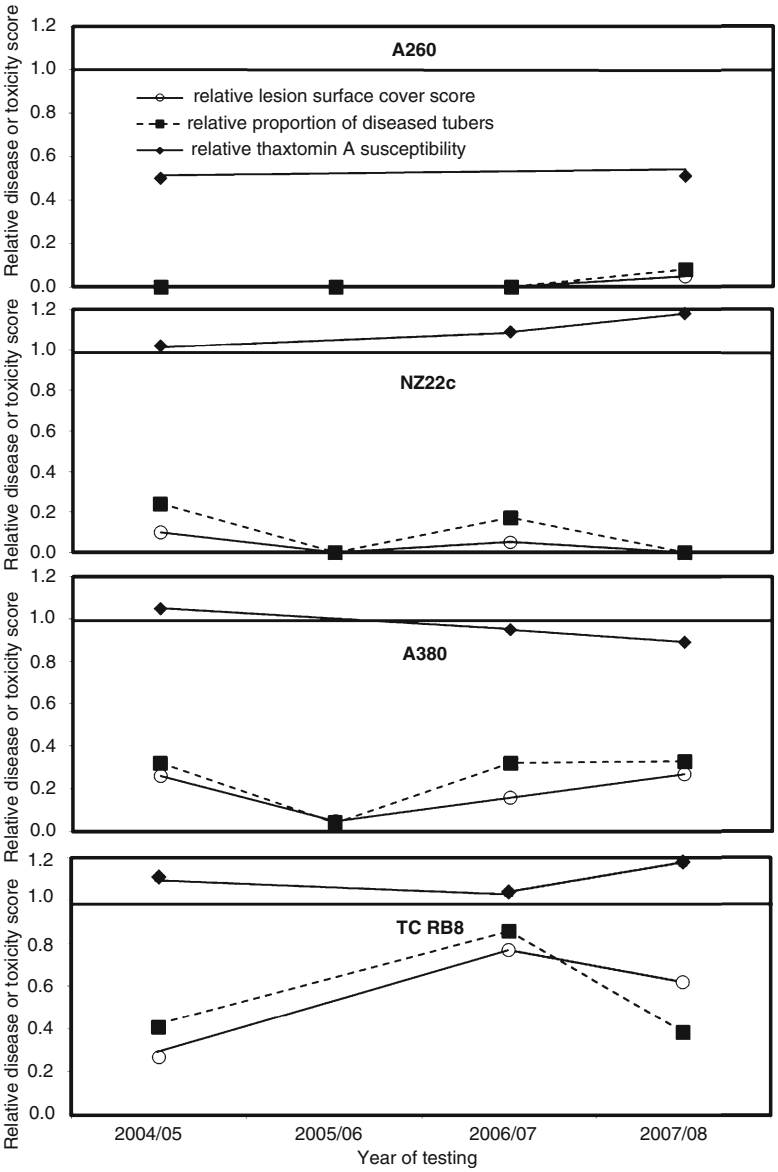

Fig. 19.3 Consistency of thaxtomin A response, and enhanced disease resistance (relative to parent cultivar (1.0)) within four somaclonal variants

then evaluated for relative yield, tuber quality and processing performance in a series of field trials. Tubers harvested from each plot were assessed for tuber yield, quality and cooking characteristics

Harvested tubers were graded into size classes and weighed. Total tuber yields per plant were calculated (tuber number and tuber mass) and compared to that

Table 19.2 Performance data for a selection of common scab disease-resistant somaclonal variants showing distinct traits relative to unselected parent cultivar

Phenotype		Increased yield	Equivalent yield	Very poor yield	
		Equivalent toxin sensitivity			Toxin tolerance
Clone name		TC-RB8	A380	NZ-22c	A260
Thaxtomin A tolerance	Leaflet bioassay	–	–	–	–
	Tuber slice bioassay	110	100	100	52
Disease resistance	Incidence (tubers with lesions)	70	18	9	3
	Severity (lesion surface coverage)	52	14	5	2
	Severity (lesion depth)	74	68	80	87
Tuber yield	Tuber number	113	112	49	76
	Tuber weight (total)	124	85	14	31
	Tuber weight (fry grade)	127	84	7	25
Tuber quality	Specific gravity	101	100	–	–
	Dry matter content	105	95	–	–
	Flesh colour	100	110	–	–
Cooking quality	Dark end (defect)	110	32	–	–
	Best fry colour (c000)	37	10	–	–
	Unacceptable fry colour (c1–2)	0	0	–	–
Tuber defects	Misshapen tubers	100	12	–	–
	Cracked tubers	21	29	–	–
	Hollow heart	824	0	–	–
	Internal browning	281	23	–	–

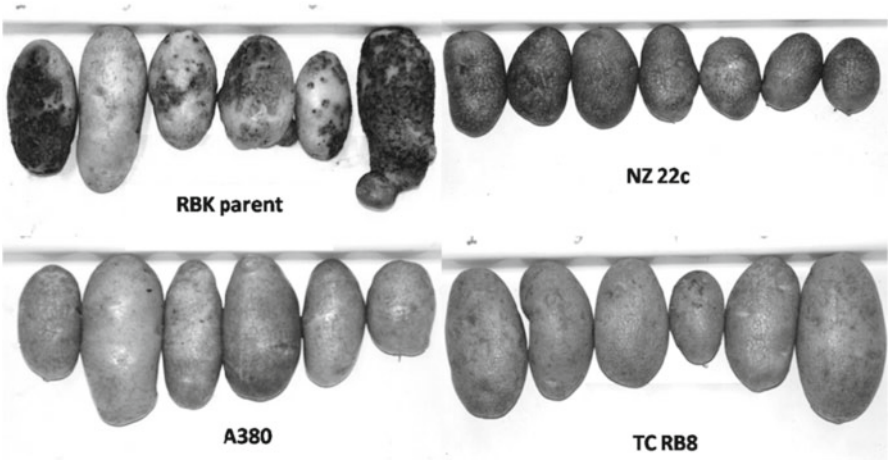

Fig. 19.4 Typical severity of common scab on unselected Russet Burbank parent cultivar and three disease resistant somaclonal variants

obtained from the parent cultivar within the same trial to give a relative yield score. Tubers with external defects (misshapen or cracked tubers) and small tubers (<75 g) were then removed and tubers reassessed to give a relative commercial (fry grade) yield assessment. Tubers were then assessed for internal defects (hollow heart and internal browning), tuber flesh colour (against a standard chart), specific gravity and dry matter content. Samples were cooked following industry standard protocols. Chip colour (using standard colour charts) and presence of dark end defects (representing sugar accumulation and subsequent caramelisation after cooking) were assessed.

Yield performance of the variants varied markedly, from very poor to equivalent or in some cases (3% of variants) better than the parent Russet Burbank cultivar (Table 19.1; Fig. 19.5). The majority of variants evaluated showed significant reductions in yield compared to the parent (68% of variants for total tuber yield, and 72% for fry grade yield) indicating co-selection of detrimental traits with disease resistance (Table 19.1; Fig. 19.5). We found a weak negative correlation between tuber yield (as assessed by weight of tubers per plant) and relative disease resistance within selected variants tested (Fig. 19.6). On average variants yielded 66% (total tuber weight) and 64% (fry grade) of the tuber mass of the parent. Tuber number was often suppressed as well (average of 36% of the parent).

However, we did identify a reasonable number of disease-resistant variants with equivalent yields to the parent cultivar, and furthermore, a few disease-resistant variants (e.g. TC-RB8; Table 19.2; Fig. 19.5) consistently yielded more tuber mass than the parent.

A few (2–6%) variants showed significantly greater tuber or cooking defects than the parent, however, the majority had equivalent or better tuber quality characteristics and cooking qualities than the parent cultivar (Table 19.1). Independent testing

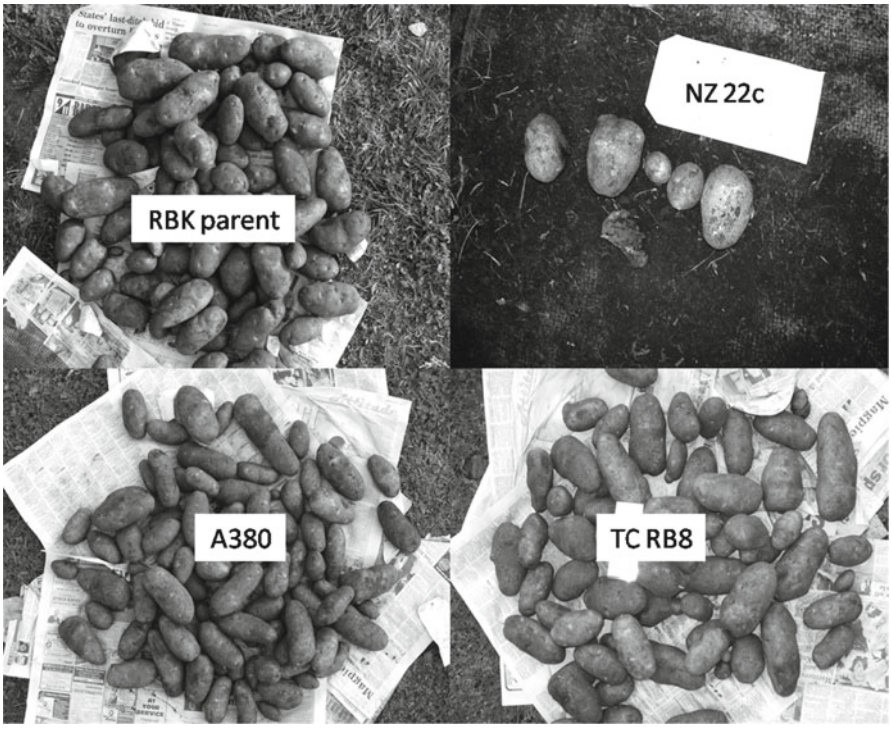

Fig. 19.5 Typical tuber yield (per plot) of Russet Burbank parent cultivar and three disease resistant somaclonal variants

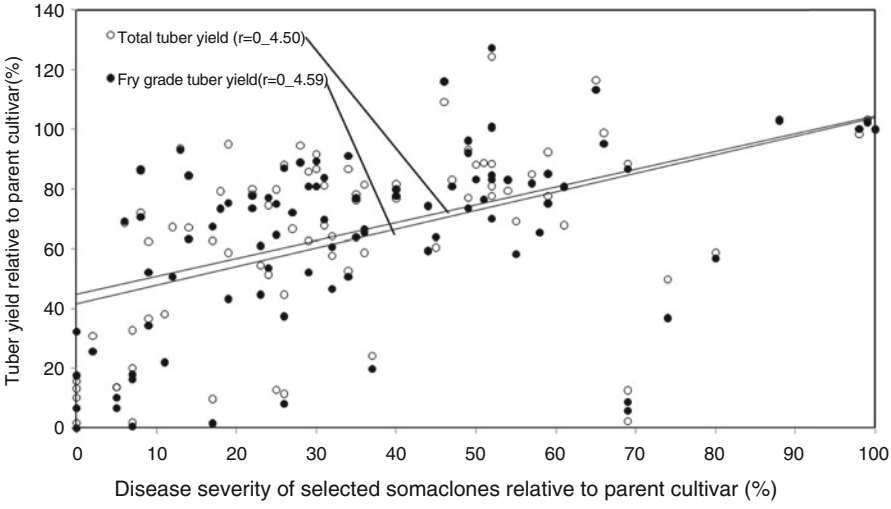

Fig. 19.6 Correlation between mean relative disease severity (tuber lesion coverage) and mean relative tuber yields (total and commercial grade weight) for the Russet Burbank variants

of a selection of the variants by a commercial French fry processing company confirmed these quality characteristics. High yielding disease resistant variants (TC-RB8 and A380; Table 19.2) have been progressed through to Plant Breeders Rights registration and are under assessment for commercial exploitation.

19.7 Possible Novel Resistance to Broad Range of Tuber-Invading Pathogens

During field assessment of the common scab resistant variants, we found that many appeared to exhibit enhanced resistance to a second important soil-borne disease, powdery scab (Fig. 19.7). This was interesting because although powdery scab produces symptoms that somewhat resemble common scab, this disease is caused by infection with a very different pathogen, the Plasmodiophoromycete *Spongospora subterranea* (de Boer 1991). The genetics, pathogenicity factors and environmental conditions favoured by this pathogen are completely different to those for the common scab pathogen. The only obvious linkage is that both pathogens penetrate developing potato tubers through immature lenticels on the tuber skin (Diriwachter and Parbery 1991; Adams and Lapwood 1978).

Lenticels are natural openings in the tuber periderm (outer skin layer) used for gas exchange (Wiggington 1973). They are recognised as important sites for pathogen invasion for many important diseases of potato. Common scab (Adams and

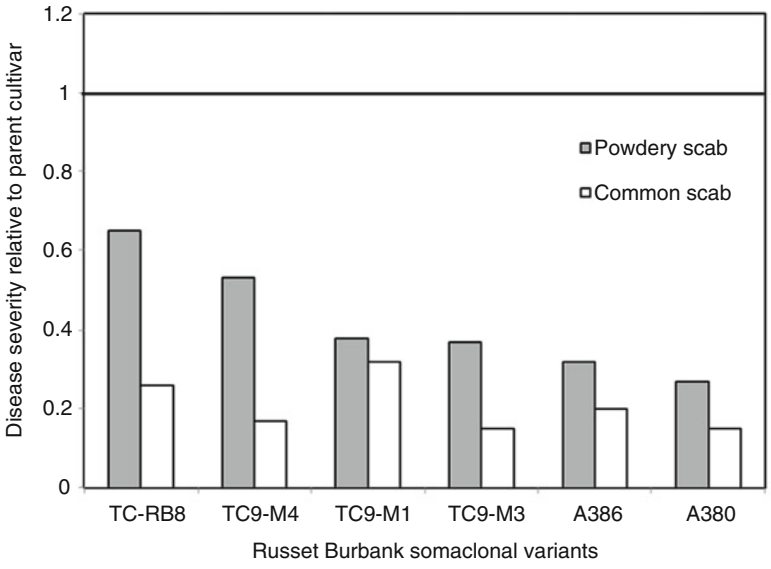

Fig. 19.7 Severity of powdery and common scab relative to the parent cultivar (1.0) for a selection of Russet Burbank somaclones (mean of five field trials)

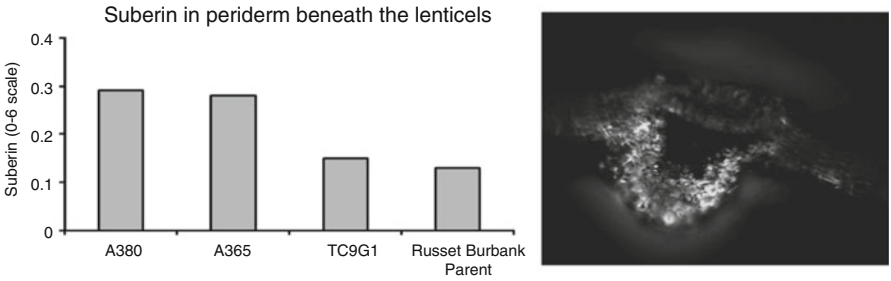

Fig. 19.8 Accumulated suberin in lenticel periderm of disease resistant (A380 and A365) and susceptible (TC9G and Russet Burbank parent) somaclones. Transverse lenticel section (A380) showing fluorescent suberin

Lapwood 1978), powdery scab (de Boer 1991; Diriwachter and Parbery 1991), tuber soft rot (Fox et al. 1971; Scott et al. 1996), and the tuber rot form of late blight (Adams 1975; Miller et al. 2002) are all examples of major tuber-infecting potato diseases associated with lenticel invasion. Potato cultivars resistant to certain tuber-invading pathogens have been characterised with low lenticel density, and/or more cell layers in the periderm and intensive suberisation and cuticularisation in lenticel tissues (e.g. Weber and Bartel 1986). For many such diseases, susceptibility of tubers to infection is also known to decrease as tubers mature. As tubers develop, lenticels mature, become filled with specialised "packing cells" and suberin and cuticular waxes are deposited within the lenticel periderm. Suberin is a cell wall lipid polyester that protects plant tissues from dehydration and is well known to provide a barrier to pathogen invasion (Kolattukudy 1977; Bernards 2002). Both packing cells and suberin are believed to assist in reducing pathogen invasion of mature lenticels (Adams 1975; Kolattukudy 1977; Miller et al. 2002).

In a preliminary study to elucidate possible physiological mechanisms of enhanced resistance to both common and powdery scab in our somaclonal variants, tubers were harvested from two disease-resistant and two susceptible somaclonal lines that had been grown in the presence or absence of the common scab pathogen (Khatri 2009). Lenticel development was studied on the harvested tubers. Whilst no differences in lenticel density or size were found, we noted that the two resistant somaclonal lines had significantly increased evidence of suberin in the periderm cell layers beneath the lenticels (Fig. 19.8). This response was dependent upon exposure to the pathogen (an inducible defence response).

We hypothesise that enhanced suberin production associated with developing lenticels on potato tubers may represent a common mechanism for enhanced resistance to common scab and powdery scab disease within our somaclonal variants. This is currently under further investigation.

Diseases associated with invasion of tubers through lenticels are common and highly damaging to potato production worldwide (Stevenson et al. 2001). The ability to use *in vitro* cell selection techniques to select for broad-scale resistance against a

suite of tuber-infecting diseases (possibly through reduced susceptibility of tuber lenticels), whilst retaining important characters of the parent cultivars, would be highly significant and desirous.

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