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Selection and regeneration of toxin-insensitive plants from tissue cultures of oats (*Avena sativa*) susceptible to *Helminthosporium victoriae* *

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Summary. Insensitivity to the pathotoxin victorin, which is produced by the fungus *Helminthosporium victoriae* (Meehan and Murphy), was selected in tissue cultures of oat (*Avena sativa* L.) lines heterozygous for the dominant sensitive allele *Vb*. The *Vb* allele imparts both susceptibility to *H. victoriae* and resistance to several races of oat crown rust (*Puccinia coronata* var. 'avenae', Fraser and E. Led.). None of 84 homozygous *Vb Vb* oat calli survived when grown on victorin-containing medium. Among 175 calli of heterozygous *Vb vb* cultures grown on toxin-containing medium, 16 representing 13 separate embryo-derived culture lines produced surviving callus sectors or shoots. Based on leaf bioassays of plants regenerated after toxin selection, nine culture lines gave toxin-insensitive plants and two gave plants showing the toxin sensitivity of the parent. Two selected lines failed to regenerate. Plants regenerated from 30 culture lines which had never been exposed to toxin-containing selection medium were all toxin sensitive. The toxin insensitivity of the regenerants from the toxin-selected culture lines was heritable since progeny of these plants were all insensitive. The toxin-insensitive selected lines all were found to have coincidentally lost the *Vb* crown rust resistance of the original line. In cytological analysis of meiotic cells of regenerants from the selected cultures, no chromosomal deficiency was found which could be associated with, and thus account for, the loss of sensitivity to the toxin. Somatic recombination and mutation to *vb vb* are other possible origins of toxin insensitivity in the selections. The victorin selection demonstrates that specific resistance can be selected in tissue cultures of oats. It also provides a highly sensitive scheme to test effects of culture conditions and chemical agents on induction of genetic and chromosomal changes in tissue cultures.

Key words: Mutant selection – In vitro selection – Toxin resistance – Crown rust resistance – Victorin

Introduction

Recovery of disease resistant plants by selection of cell cultures resistant to a toxin produced by a pathogen represents a direct application of cell culture approaches to crop improvement. Selections in plant cell cultures for pathotoxin resistance include resistance in potato (*Solanum tuberosum*) to culture filtrates of *Phytophthora infestans* (Behnke 1980a) and *Fusarium oxysporum* (Behnke 1980b), in tobacco (*Nicotiana tabacum*) to toxins from *Pseudomonas syringae* and *Alternaria alternata* (Thanutong et al. 1983), and in *Brassica napus* to toxin of *Phoma lingam* (Sacristan 1982). In grasses Gengenbach and coworkers (1975) recovered *Helminthosporium maydis* toxin-resistant plants from cell cultures of T-cytoplasm *Zea mays* and Heinz and coworkers (1977) selected *H. sacchari* resistance in sugarcane (*Saccharum* sp.) cultures. In maize, *H. maydis* toxin resistance is controlled through the cytoplasm as a maternally inherited trait. The inheritance of resistance to *H. sacchari* toxin in sugarcane, a polyploid, is not known.

The victorin system in oats (*Avena sativa*) has several features which are unique from these other toxin systems. First, the victorin pathotoxin produced by the fungus *H. victoriae* (Meehan and Murphy) inhibits growth only in lines of oats which have the

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dominant nuclear allele *Vb*. This system thus can be used to address questions such as whether a recessively-inherited resistance trait can be selected in disomic tissue in culture and what types of resistant lines will be recovered when heterozygous toxin-sensitive cultures are used in selection. Secondly, the toxin is highly specific. Resistant *vb vb* lines of oats are tolerant to a toxin level 100,000 times higher than the level giving 50% root growth inhibition in sensitive lines (Luke and Wheeler 1955), thus selection can be made quite stringent. Finally, the *Vb* gene has a coincident trait of providing resistance to several races of oat crown rust (*Puccinia coronata* Cda. var. 'avenae', Fraser and E. Led.).

The *Vb* gene, first identified in the oat cultivar 'Victoria', was introduced into many breeding lines in the 1940's as a source of crown rust resistance. Recognition that the *Vb* gene also causes sensitivity to *H. victoria* occurred only after lines carrying the *Vb* gene were widely grown (Meehan and Murphy 1946). Drastic losses occurred in the U.S. oat crop in 1946 and 1947 due to widespread *H. victoriae* infection, and all lines with the *Vb* gene were dropped from oat breeding programs. Since then there have been several unsuccessful efforts to separate the crown rust resistance from the Victoria blight sensitivity either by recombination or mutagenesis (Wheeler and Luke 1955; Wallace and Luke 1961).

The *Vb* gene is inherited as a Mendelian disomic dominant even though the cultivated oat *A. sativa* is an allohexaploid (2n=6x=42). Temperature-sensitive and intermediate-resistance mutants have been recovered following mutagenesis and selection among M_2 seedlings (Luke and Wallace 1969). Both the toxin reactions and the crown rust reactions are temperature sensitive or intermediate in these mutants. Homozygotes (*Vb Vb*) and heterozygotes (*Vb vb*) are nearly indistinguishable in current toxin bioassays. The reactions of the hemizygote and the null state are unknown since lines of this type have never been identified.

This report describes selection for toxin-insensitivity in tissue cultures of *Vb Vb* homozygous and *Vb vb* heterozygous oats sensitive to *H. victoriae* toxin. Plants regenerated from selected cultures and the progeny of these plants were then characterized for reaction to victorin and to crown rust and analyzed cytologically. Further uses of the victorin system in testing the effects of culture conditions and chemical agents on the induction of chromosomal and genetic variation in culture are proposed.

Materials and methods

Plant material

Oat lines placed into culture included two *Vb Vb* victorin-sensitive, crown rust-resistant *A. sativa* cultivars, 'Victorgrain' and 'Victoria', and *Vb vb* hybrids from the cross GAF×'Victoria'. GAF is an F_4-derived line from a cross of the *A. sativa* cultivar 'Garland' and a wild oat *A. fatua* 1223 with selection in the F_2 and F_4 generations for tissue culturability and for cultivated plant and seed type. *A. fatua* 1223 had been found earlier to be highly culturable (Rines and McCoy 1981). GAF has proven superior in frequency of regenerable culture initiation compared to all *A. sativa* cultivars tested. The GAF/'Victoria' embryos used to initiate cultures for selection were obtained following manual emasculation and pollination while the homozygous embryos came from natural self-pollinations.

Victorin preparation

An isolate of *H. victoriae* selected for high toxin production in culture was grown as previously described (Luke and Wheeler 1955). The toxin was purified by precipitating the culture filtrate with 50% methanol at 5 °C. The precipitate was discarded, the methanol removed with a rotary vacuum evaporator, and the supernatant lyophilized. This material was extracted with cold methanol and brought to dryness in a rotary vacuum evaporator. The residue that contained the toxin was rehydrated and placed on acid alumina columns (3.0×7.5 cm) packed in hexane. The columns were washed with hexane, benzene, diethyl ether and methanol. Victorin was eluted from the column with a 2N formic acid – 50% methanol solution. Methanol and formic acid were removed using a rotary vacuum evaporator and the supernatant was lyophylized. Additional purification was carried out with a Bio-Gel P-4 (Bio-Rad, Richmond, CA, USA) column[1] (1×32 cm, exclusion limits 3,600 mw). Toxin-containing fractions from this column were lyophylized. This material contained 10,500 units of toxin per ml. A unit of toxin is defined as 1,000 X the amount giving 50% inhibition of seedling root growth (Luke and Wheeler 1955).

Culture initiation, selection and regeneration

Oat tissue cultures were initiated from 10- to 12-day-old embryos plated onto MS medium (Murashige and Skoog 1962) containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and maintained on the same medium by monthly subculture (Rines and McCoy 1981). Selection began approximately 5 months after initiation by transferring cultures onto MS medium containing 1 mg/l 2,4-D and a predetermined "marginally lethal" level (5 units toxin per ml) of victorin. After 45 days, growing culture sectors or shoots were transferred to regeneration medium consisting of MS medium with 2 mg/l α-naphthaleneacetic acid (NAA) and 0.2 mg/l 6-benzylaminopurine. Developing shoots were transferred to 250-ml Erlenmeyer flasks containing 50 ml of MS medium plus 2 mg/l NAA. Resulting plantlets were placed in soil. A non-selected group of cultures and regenerants were produced by similar procedures except the cultures were never exposed to toxin-containing medium.

Analysis of regenerated and progeny plants

Developing panicles were collected at microsporogenesis and fixed in 3:1 (95% ethanol:glacial acetic acid) for meiotic analysis. At the same time, the terminal 15–20 cm portion of the youngest and the second youngest leaves were taken for toxin-reaction bioassay (Luke et al. 1966). Leaf samples were recut under water, and cut ends placed in 5-ml vials con-

[1] Mention of a trademark or propriety product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable

taining 3 ml of victorin solution (10 units of toxin per ml). After 2–3 days in the light at room temperature, the leaf samples were tested for the severe wilting response characteristic of toxin sensitive leaves. Leaf samples of parental and *Vb vb* heterozygous plants served as checks. Sample leaves in water served as controls.

Crown rust reactions of progeny were tested by P. C. Rothman, USDA-ARS Cereal Rust Laboratory, St. Paul, MN (Roelfs and Rothman 1971). Seven-day-old seedlings were inoculated with an oil suspension of urediospores of race 202 and placed in a dew chamber overnight. After 14 days growth in the greenhouse, the plants were scored for rust reactions.

Results

Selection in homozygous Vb Vb toxin-sensitive cultures

Twelve of 65 immature embryos of the cultivar 'Victorgrain' and two of 21 embryos of 'Victoria' plated on tissue culture initiation medium developed sufficient regenerable-type calli over a period of 5 months to attempt selection. There were approximately 6 callus pieces per embryo-derived line. Thirty days after transfer to a victorin-containing medium there were no visible regions of tissue growth or survival in any of these calli.

Selection in heterozygous Vb vb toxin-sensitive cultures

Vigorous tissue culture lines were attained over a 5 month period from 22 of 38 *Vb vb* immature embryos plated. A total of 175 calli from these culture lines were transferred to a toxin-containing selection medium. After 45 days, 13 culture lines (16 calli) had surviving callus sectors or shoots (Fig. 1). When these surviving tissues were transferred to regeneration medium, plants were obtained from 11 of the culture lines. The number of regenerated plants ranged from a single plant in lines where only a small sector had survived selection to multiple plants where there had been vigorous callus growth on the selection medium.

Each regenerated plant (R_0) was tested for victorin resistance using a leaf bioassay (Fig. 2). In this bioassay leaves of control *Vb Vb* and *Vb vb* plants gave a marked wilting response within two days. Regenerated plants from 9 toxin-selected culture lines proved to be toxin insensitive while plants from two lines were toxin sensitive. These two toxin-sensitive regenerants were obtained from cultures in which the only surviving tissue had differentiated into small shoots (Table 1). These shoots may have been escapes from the toxin selection. The more differentiated tissues in the morphologically mixed cultures tended to survive toxin selection longer, possibly due to a reduced uptake or penetration of the toxin. Also, selection was made at a marginally lethal level of toxin. In all the culture lines in which there was vigorous callus growth on the selection medium, the regenerated plants were all insensitive

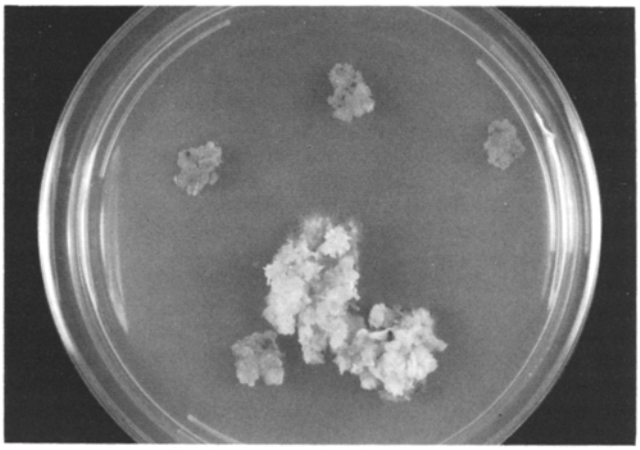

Fig. 1. Calli (2 growing, 4 inhibited) 45 days after transfer onto toxin containing medium. These calli had been initiated from an *H. victoriae* toxin-sensitive *Vb vb* GAF/Victoria embryo

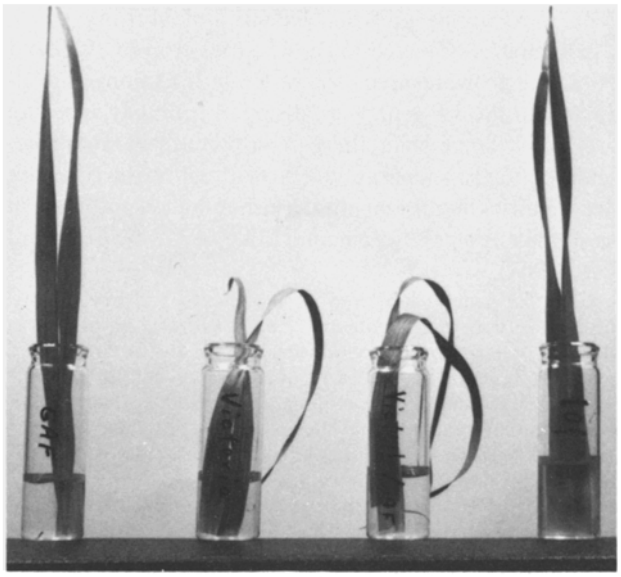

Fig. 2. Leaf bioassays for *H. victoriae* toxin reaction showing response three days after cut leaves were placed in toxin solution. (1. to r.) 'GAF' *vb vb* insensitive check, 'Victoria' *Vb Vb* sensitive check, *Vb vb* sensitive line as used to initiate tissue culture, insensitive regenerant from toxin-selected culture

(Table 1). Among regenerants from 30 culture lines never exposed to toxin-containing medium, all were toxin sensitive.

The inheritance of the victorin insensitivity of the regenerants from the toxin-selected cultures was analyzed by testing the progeny (R_1 generation) of these regenerants. Only insensitive progeny were obtained from victorin-insensitive R_0 plants (Table 1). In four of the lines none of the regenerated plants set seed so progeny tests were not possible. Sterility in regenerated oat plants is common (McCoy et al. 1982), and several

Table 1. Tissue survival following 45 days growth on toxin-containing medium and characterization of regenerated plants and their progeny in 22 5-month-old culture lines initiated from *H. victoriae* toxin (victorin) sensitive, crown rust resistant *Vb vb* GAF/Victoria oat embryos

Culture line designation	Callus tissue on victorin medium		Regenerated plants	Progeny plants	
	No. calli plated	Surviving tissue	Victorin response[a]	Victorin response[a] (I : S)	Crown rust reaction[b] (Res. : Susc.)
101	12	1 large callus sector	I	6 : 0	0 : 6
102	12	1 small shoot	S	4 : 15	15 : 4
103	12	1 small callus sector	I	–	–
104	6	1 small callus sector	I	–	–
105	6	1 large callus	I	12 : 0	0 : 12
106	12	1 large callus	I	15 : 0	0 : 15
107	12	1 small callus sector	–	–	–
108	6	1 small shoot	S	5 : 18	18 : 5
109	6	2 large calli	I	–	–
110	6	2 large calli	I	8 : 0	0 : 8
111	6	2 large calli	I	9 : 0	0 : 9
112	6	1 small callus sector	I	–	–
113	6	1 small callus sector	–	–	–
114–122	67	None	–	–	–
Total	175	16	9 I lines, 2 S lines	5 I lines, 2 segregating lines	5 Susc. lines, 2 segregating lines

[a] Response to the toxin victorin in leaf bioassays: I = Insensitive, S = Sensitive, – = no plants regenerated or seed set

[b] Reaction to crown rust inoculation: Susc. = Fully susceptible, Res. = Resistant, – = no seed set on parent plant

of the developing panicles had been sacrificed for meiotic chromosomal analysis. Progeny from victorin-sensitive R_0 plants segregated in a ratio of about three sensitive: one resistant, as did progeny of the *Vb vb* parent line.

Because crown rust resistance is expressed along with victorin sensitivity as an effect of the *Vb* allele, progeny of selected victorin insensitive plants were tested for their reaction to crown rust. All gave a fully susceptible reaction (Table 1). Loss of sensitivity to victorin thus resulted in a coincident loss of crown rust resistance, just as had occurred in other selection approaches (Wheeler and Luke 1955; Wallace and Luke 1961).

When meiotic cells of regenerated plants from the nine insensitive selections were examined, only selection 109 had evident chromosome loss. Because the plants of this line were sterile, however, we could not test if this chromosome loss was associated with the loss of victorin sensitivity.

Discussion

Selection for the recessive trait of victorin insensitivity was highly effective in heterozygous *Vb vb* oat tissue cultures. This result is an additional example of successful selection for a trait in the relatively complex, partially differentiated type of cereal tissue cultures often needed to maintain regeneration capacity. Selection in *Vb Vb* homozygous lines was not successful. This latter result is as expected if mutation or loss of both *Vb* alleles is required for insensitivity.

Selection appears to have played a significant role in the recovery of toxin-insensitive plants from the *Vb vb* oat cultures since no toxin-insensitive plants were recovered among regenerants from 30 independently-initiated culture lines not subjected to toxin selection. This situation contrasts to reports of occasionally frequent recovery of toxin-resistant regenerants from unselected cultures for *H. maydis* resistance in maize (Brettell et al. 1980; Umbeck and Gengenbach 1983) and *H. saccari* resistance in sugarcane (Heinz et al. 1977; Larkin and Scowcroft 1983). The differences in frequencies of toxin-resistant regenerants from unselected cultures among the oats, maize, and sugarcane systems is probably due to the genetic nature of the resistances. *H. victoriae* sensitivity in oats appears to be under the control of a single nuclear locus. *H. maydis* sensitivity in maize is under cytoplasmic control. *H. sacchari* sensitivity in sugarcane behaves as a quan-

titative trait, and chromosome number is polyploid in sugarcane and often variable among tissue culture regenerants.

The frequency and timing with which toxin insensitivity originated in the toxin-selected oat tissue cultures is difficult to ascertain. In 3 of the 9 culture lines which eventually gave rise to toxin-insensitive regenerated plants, there was vigorous callus growth in 2 of the 6 calli initially subcultured onto the toxin-containing medium (Table 1). These two calli could represent either independent mutations or a single mutation occurring before the culture was split during subculturing. The occurrence of more than one independent event giving toxin insensitivity in a culture line would not be unlikely judging from the high frequency of culture lines (9 of 22) in which insensitivity was recovered.

The genetic basis of the toxin insensitivity selected in *Vb vb* oat tissue cultures is not known although there are several possibilities. The loss of the chromosome or chromosome segment on which the dominant *Vb* allele is located may give toxin insensitivity. Identification of the chromosome involved also would physically map the *Vb* gene in oats. A high frequency of chromosome breakage and partial chromosome loss has been reported in plants regenerated from oat tissue cultures (McCoy et al. 1982). When meiotic cells of regenerated plants from five toxin-selected regenerable cultures were analyzed in this study, however, no cytogenetic alterations could be specifically associated with loss of toxin sensitivity. An alternative origin of resistance in these selected lines is that somatic recombination in a *Vb vb* culture would produce homozygous recessives. Lorz and Scowcroft (1983) reported a greatly enhanced frequency of twin spots in *Su su* plants of *Nicotiana tabacum* which had been regenerated from protoplast cultures. This increase apparently was due to an enhancement of somatic crossing-over brought about by the culture process. We had no ready means to monitor the possible occurrence of somatic crossing-over, either by recognition of twin spots or by flanking markers, but we consider somatic recombination a likely origin of many of the insensitive cultures selected. Mutations in the *Vb* locus affecting the *Vb* product or level of expression of the *Vb* locus represent a further possible origin of insensitive selections. Variants that are partially sensitive to the toxin, as found by Luke and Wallace (1969) following radiation of seeds, would be possibilities of this type.

Selection for insensitivity to victorin was highly effective in *Vb vb* heterozygous sensitive lines of oats, but insensitivity was not obtained from *Vb Vb* homozygous dominant culture lines. Because we were unable to determine either the origin or the frequency on a cellular basis of the appearance of insensitive sectors in *Vb vb* cultures, we could not predict an expected frequency for the recovery of insensitives in *Vb Vb* cultures. For example, somatic recombination, which is a likely source of insensitives in the heterozygous cultures, could not produce double recessives in a homozygous *Vb Vb* culture unless it was preceded by a mutational event. Presumably two mutations, a mutation followed by somatic recombination, or a mutation either preceded or followed by a chromosome loss would be necessary for expression of victorin insensitivity in *Vb Vb* lines. Lorz and Scowcroft (1983) reported that both prolonged cell culture and chemical mutagenesis enhanced the frequency of apparent somatic crossing-over in protoplast cultures of *Su/su* heterozygotes of *Nicotiana tabacum*. Genotype choice and prolonged culture in oats (McCoy et al. 1982) and the inclusion in the culture media of chemicals such as griseofulvin in alfalfa cultures (Schiavo et al. 1980) have enhanced the frequencies of chromosomal losses in cultures. Perhaps the incorporation of these approaches would enhance the opportunity for selection of recessively inherited traits in homozygous dominant disomic cultures.

The victorin tissue culture selection system in oats has many attractive features for further studies, both in investigating the *Vb* locus itself and as a model tissue culture selection system in cereals. The *Vb* locus with its dual effects of crown rust resistance and *H. victoriae* susceptibility is an interesting tool for analyzing complex host-pathogen interactions for two unrelated pathogens. *Vb* is a relatively well characterized nuclear gene system in which toxin resistance is recessive. This tissue culture selection scheme thus can be used to test effects of chemical agents and culture condition on induction of mutations, somatic recombination, and chromosome loss – all largely uncharacterized factors with potential major effects on the use of tissue cultures in plant somatic cell genetic manipulations.

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