The Development of Asparagus Somaclones with High Levels of Resistance to *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum*

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ABSTRACT

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Asparagus somaclones were produced by subculturing shoots initially generated from callus-derived protoplasts of *Asparagus officinalis* cv. Lucullus 234 through callus cycles for more than a 3-year period. One hundred twenty protoplast-derived somaclones of Lucullus 234 were screened for resistance to two virulent Michigan isolates of *Fusarium oxysporum* f. sp. *asparagi* (FOA10 and FOA50) and *F. proliferatum* (FM12 and FM49) in the greenhouse. Somaclones had significantly ($P < 0.05$) more resistance to the *Fusarium* spp. than did the vegetatively micropropagated plants of the parental cultivar Lucullus 234. Of the somaclones that contained higher levels of disease resistance, a minimum of 12 micropropagated plants from each were produced and rescreened for resistance to the most virulent isolate (FOA50) among the four isolates tested in the greenhouse. Two somaclonal lines, R7 and R4, were highly significantly ($P < 0.01$) more resistant to the FOA50 of *F. o.* f. sp. *asparagi* than were the vegetatively micropropagated parental plants. No morphological or growth differences were observed between these lines and the parental cultivar.

Additional keywords: asparagus decline, tissue culture

Asparagus officinalis L. is an economically important vegetable crop that is produced worldwide (34). However, asparagus decline syndrome, primarily caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *asparagi* S.I. Cohen & Heald and *F. proliferatum* (T. Matsushima) Nirenberg (syn. *F. moniliforme* J. Sheld.), occurs throughout the asparagus-growing regions of the world and is the major limiting factor in asparagus production (3,12,19,20,47). The disease syndrome is characterized by reduced production resulting from declining plant densities and reduced size of spears (40). Asparagus decline causes loss in longevity and productivity of established fields, and causes difficulty in replanting asparagus where it was previously grown. These characteristics, of course, decrease annual yields of asparagus over time. The use of conventional methods, including chemical and cultural methods for controlling *Fusarium* spp., has been limited (24,41,42). Development of resistant cultivars appears to be the most viable long-term strategy for control, but no resistant cultivars have been developed by traditional breeding methods (11,43). In addition, when traditional breeding methods are used, *A. officinalis* has low regenerative potential (34), and the development of new cultivars requires many years because of its perennial nature. It is likely that genetic diversity among the asparagus cultivars in North America is low (11,17,27).

Genetic variability has been detected from tissue, cell, and protoplast cultures, especially on periodic subculturing for various morphological and genetic changes such as polyploidy, aneuploidy, chromosome breakage, deletion, translocation, gene amplification, inversions, mutations, etc. (4,28,32). Somaclonal variation was first proposed as a novel source of agriculturally useful variation for asexually propagated crops such as sugarcane (22) and potato (36). Agriculturally useful somaclonal variants have been identified that have desirable traits such as increased solids in tomato, male sterility in tomato and rice, higher yields and enhanced protein production in rice, earliness in maize, freezing tolerance in wheat, and increased sugar content in sugarcane (1,14).

Development of resistance to fungal, bacterial, and viral diseases in various crops has been the major contribution of somaclonal variation (25). In alfalfa, somaclones resistant to *F. oxysporum* were regenerated (21). Screening of alfalfa somaclones that were regenerated from protoplasts resulted in plants that were resistant to *Verticillium albo-atrum* Reinke & Berthier (26). Two somaclonal lines of rice were resistant to sheath blight in the field (45). Daub (8) reported that protoplast-derived somaclones of tobacco cultivars increased resistance to several major tobacco pathogens, including tobacco mosaic virus, *Meloidogyne incognita* (Kofoid & White) Chitwood, and *Phytophthora parasitica* var. *nicotianae* Tuck., and identified three somaclones whose progeny had enhanced resistance to black shank (*P. p.* var. *nicotianae*) in both field and greenhouse tests. Resistance in celery to *Fusarium oxysporum* f. sp. *apii* race 2 was enhanced with somaclonal variation (46). Somaclonally derived resistance to tomato mosaic virus and tobacco mosaic virus has been obtained in tomato (38). Among six cultivars or varieties derived from somaclonal variation to date (13,23,29,30,37), two possess enhanced disease resistance. The sugarcane cv. Ono is higher yielding and shows increased resistance to Fiji disease (23). DNAP 17, a tomato variety, shows resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 2 (13).

A cultivar of *A. officinalis*, Lucullus 234, had relatively higher resistance to virulent Michigan isolates FOA10 of *F. oxysporum* and FM12 of *F. proliferatum* among 90 cultivars and breeding lines of this species tested (39). A previous study screened somaclones of two cultivars of *A. officinalis*, Lucullus 234 and Jersey Giant. Somaclones of Lucullus 234 exhibited a higher level of resistance to *F. oxysporum* and *F. proliferatum* than did the Jersey Giant somaclones (M. L. Smither and C. T. Stephens, *unpublished*). We undertook this survey to determine if somaclonal variation could generate a high level of resistance to *Fusarium* spp. from a moderately resistant asparagus cultivar such as Lucullus 234.

MATERIALS AND METHODS

Production of somaclones. Because of the previously reported higher resistance of Lucullus 234 to *Fusarium* spp. (39) and the higher probability of getting variants from plants regenerated from protoplasts than from other types of culture (such as callus, stems, somatic embryos, and adventitious buds [9]), initial shoots regenerated from callus-derived protoplasts of Lucullus 234 (7) were used to produce somaclones. These shoots were cultured on Murashige and Skoog (MS) medium

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(31) that was supplemented with 6-BAP at 0.1 mg/liter (RM17). Calli formed on the shoots within 15 to 30 days, and new shoots developed on the calli within 30 days. Because accumulation of somaclonal variability has been positively correlated with the duration of the culture (16), this technique was used to maintain shoot production for more than 3 years, and the produced shoots were used as the somaclonal source. Effects of different concentrations and combinations of root-inducing agents such as auxins (NAA, 2,4-D, IAA, and IBA), ancymidol, and sucrose were examined on root differentiation. Rooted plants were cultured in a hormone-free MS medium for shoot and root elongation for another month. All cultures were placed at 27°C with 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ light and a 16-h photoperiod. All rooting experiments contained at least 12 replicates per treatment and were conducted at least three times.

Before plants were transferred to the greenhouse, they were acclimatized to greenhouse conditions. Plants were transferred to pots containing a synthetic medium (vermiculite:peat:perlite, 1:1:1). The pots were placed in a plastic bag at 22 to 24°C under cool-white fluorescent light and a 24-h photoperiod. The bags were completely sealed for the first week, punctured with three holes for the second week, and opened for the third week. The pots were maintained one additional week after the plastic bag was completely removed at the end of the third week, then were moved to the greenhouse.

Vegetative micropropagation of Lucullus 234. Since asparagus is a cross-pollinated plant, it has a high degree of natural heterozygosity (34), and the considerable genetic variations of several traits in asparagus cultivars are well recognized (44). Also, due to the limited number of Lucullus 234 seeds, it was necessary to develop a protocol of vegetative micropropagation to obtain the homozygous parental Lucullus 234 plants for use as a control. Two different concentrations of cytokinin 6-BAP were tested for shoot production. Spears of the asparagus plants were surface-sterilized (7) and cut into segments, each with a single node. Two to four segments were cultured on 20 ml of either one of two different modified MS media supplemented with BAP at 0.5 mg/liter (RM18) and 0.1 mg/liter (RM17) in 25 × 150 mm culture tubes. The effect of four different auxins, IAA, IBA, NAA, and 2,4-D, on root induction was tested. Branched shoots at the node region were excised after a 15-day culture in RM17 medium. Two shoots were cultured in a culture tube with 20 ml each of four different root-inducing media containing basic MS medium, and IAA, IBA, NAA, and 2,4-D, respectively, each at a concentration of 2 mg/liter. IBA resulted in the highest root production. Six concentrations of IBA, 2, 3, 5, 7, 9, and 11 mg/liter, were tested for improving rooting efficiency. Rooted plants were cultured on a hormone-free MS medium for further shoot and root development for 1 month. All cultures were maintained at 27°C with 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ light and a 16-h photoperiod. Plants were transferred into the greenhouse after acclimatization as described above. All experiments contained at least eight replicates per treatment and were repeated four times.

Screening somaclones for resistance to *Fusarium*. One hundred twenty somaclones and 120 vegetatively micropropagated plants of Lucullus 234, generated as described above, were used for screening tests. Two Michigan virulent isolates each of *F. oxysporum* (FOA10 and FOA50) and *F. proliferatum* (FM12 and FM49) from the collection of M. L. Lacy of Michigan State University were stored according to a method described by Nelson et al. (33). Previous work demonstrated that a cultivar of *A. officinalis*, UC 157, was highly susceptible to *F. oxysporum* and *F. proliferatum*, and a cultivar of an ornamental asparagus species *A. sprengeri* Regel 'Sprengeri' was highly resistant (39). One hundred twenty plants each of UC 157 and Sprengeri were used as susceptible and resistant controls, respectively, for the screening test.

Seeds of *Asparagus* spp. were surface disinfected and germinated according to the method of Stephens and Elmer (40). One week later, germinated seeds were transferred into pots containing synthetic medium as described above and grown in the greenhouse. Inocula of FOA10, FOA50, FM12, and FM49 were prepared by the method of Wacker et al. (48). Each *Fusarium*-infested soil and the *Fusarium*-free soils (control soils) were amended with 500 g of sterile sandy loam soil, 10 g of dried asparagus fern, and 25 ml of 0.025 M asparagine in trays; and the soil mixes were autoclaved for 1 h on two consecutive days. Fifty ml of conidial suspension of each of the four isolates grown in potato-dextrose broth at 10^6 conidia/ml was added to the sterile soil mix. The sterile soil mix in trays was covered with aluminum foil and incubated for 10 days at 30°C. One-month-old seedlings of UC 157 and Sprengeri, and one-month-old greenhouse-grown somaclones and vegetatively micropropagated plants of Lucullus 234 were transferred to 53 × 28 × 6 cm trays containing 7 kg of the *Fusarium*-infested soil and control soil, respectively. Plants were fertilized (Peters 20N-20P-20K, 200 ppm) once per week.

Plants were harvested after 1 to 4 months, depending on the temperature and humidity. Disease incidence was assessed by a visual rating scale (39,48): 1 = healthy plant, no evidence of disease (highly resistant); 2 = few root lesions, less than 25% diseased (resistant); 3 = moderate number of root lesions, less than 50% diseased (moderately susceptible); 4 = many root lesions, less than 75% diseased (susceptible); and 5 = all roots flaccid or dead (highly susceptible). Experiments were designed as a two-factorial experiment using a randomized complete block design with *Fusarium* spp. as factor A and germ plasm entries as factor B, which is a split plot on A. All experiments were conducted at four different times and arranged on greenhouse benches under a natural photoperiod at 25 to 30°C. Data were analyzed by MSTAT program for ANOVA.

Stability of resistance to *Fusarium* in the more resistant somaclones. To determine if the resistance in the individual somaclones with higher levels of resistance selected from the first cycle remains stable, we maintained somaclones with disease ratings of 1 and 2 in the greenhouse for further disease screening. These somaclones were vegetatively micropropagated by the method described above except that RTM30 medium was used for root induction. At least 12 micropropagated plants from the resistant somaclones were rescreened for resistance to FOA50 as described above. FOA50 was chosen as the screening agent in these tests because FOA50 was found to be the most virulent to vegetatively micropropagated Lucullus 234 parental plants among the four isolates tested. All experiments were conducted at two different times as a randomized block design in the greenhouse under the same conditions as described above. Data were subjected to a Student's *t* test.

RESULTS AND DISCUSSION

Production of somaclones. Fifteen to 30 days after initial shoots were cultured on RM17 medium, calli were produced on the shoots, and new shoots developed on the calli within 30 days. Shoot production was maintained for more than 3 years by this procedure. When the produced shoots were transferred into different root-inducing media, shoots formed a small, compact mass resembling a crown where thick roots were attached, and then roots formed from the mass. The highest average root production (55.5%) was obtained from shoots cultured 4 weeks in an MS medium containing IBA at 11 mg l^{-1} (data not shown). Further root development was much quicker when shoots with induced roots were cultured in a hormone-free MS medium, as opposed to shoots cultured in initial root-induction media. This indicated that IBA, which is necessary for root induction, inhibited further root growth. Rooting of shoots regenerated from protoplasts in this study was not improved by ancymidol or by increasing the concentrations of sucrose in contrast to the results reported by Chin (2) and Desjardins et al. (10) (data not shown). Two hundred plants were transferred into the greenhouse; 80.5% of them survived and were used as a source of somaclones.

Vegetative micropropagation of Lucullus 234. Shoots (1 to 2 cm in length) developed from more than 90% of nodes of spears 15 days after single-node segments were cultured on RM17 medium. Roots appeared usually 15 to 30 days after produced shoots were cultured in root-inducing media. Among four different auxins tested, IBA produced the highest rooting of 33.3% in 1 month (Fig. 1). The auxin 2,4-D completely inhibited root induction (Fig. 1). The optimal concentration of IBA in the RTM 26 medium was 3 mg/liter and yielded 57.5% as the highest average for root production 1 month after culture (Fig. 2). One month after culture in RTM26, all the plantlets had well-developed crowns with four to seven spears and vigorous roots. At this stage, plants were transferred to a hormone-free MS medium for further shoot and root development for 1 month. Two hundred fifty-seven plants were transferred to the greenhouse after acclimatization as described above, and 84.4% of them survived.

Screening of somaclones for resistance to *Fusarium*. For the first cycle of screening somaclones for resistance to *Fusarium*, 120 somaclones of Lucullus 234 and 120 plants of vegetatively micropropagated Lucullus 234 parental plants, Sprengeri, and UC 157 were screened for resistance to the two isolates (FOA10 and FOA50) of *F. oxysporum* and the two isolates (FM12 and FM49) of *F. proliferatum*.

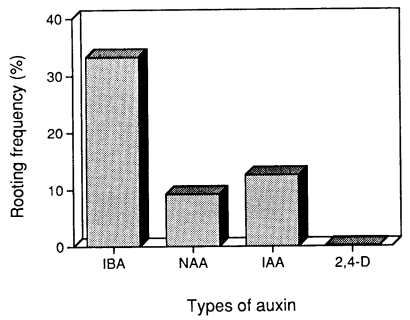

Fig. 1. Effects of different auxins (IAA, IBA, NAA, and 2,4-D), each at a concentration of 2 mg/liter, on rooting frequency (%) of the shoots produced on RM17 medium of asparagus cv. Lucullus 234 after 4 weeks in culture.

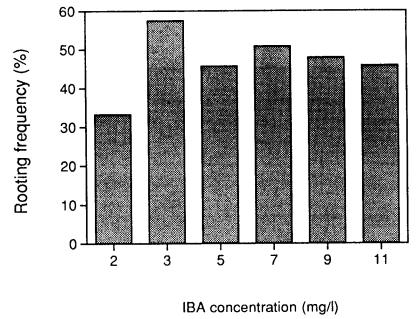

Fig. 2. Effects of IBA concentrations on rooting frequency (%) of the shoots produced on RM17 medium of asparagus cv. Lucullus 234 after 4 weeks in culture.

Both *Fusarium* spp. and asparagus germ plasm had a significant ($P < 0.05$) effect on disease incidence, but there was no significant interaction between them. FOA50 was significantly more virulent to all plants when compared to FOA10 (data not shown). Somaclones were significantly more resistant to both *Fusarium* spp. than were the vegetatively micropropagated Lucullus 234 parental plants (Table 1). Of the 120 somaclones tested, 24.2% of them (vs. 8.3% of the parental plants) fell into rating scale 1, 40.8% (vs. 41.6% of the parental plants) into rating scale 2, 15.8% (vs. 25.8% of the parental plants) into rating scale 3, 8.3% (vs. 10.8% of the parental plants) into rating scale 4, and 10.8% (vs. 13.3% of the parental plants) into rating scale 5 (Table 1). Of 120 plants of Sprengeri, 50.8% of them fell into rating scale 1, 47.5% in rating scale 2, 1.6% in rating scale 3, and none in rating scales 4 and 5. Of 120 plants of UC 157, none of them fell into rating scale 1, 17.5% in rating scale 2, 40.8% in rating scale 3, 27.5% in rating scale 4, and 14.2% in rating scale 5 (Table 1). Ninety-two percent of the somaclones and vegetatively micropropagated plants of Lucullus 234 and Sprengeri remained healthy when they were planted in noninfested soils (control soils), and 75% of UC 157 were healthy. The rest of the plants had a few root lesions (at rating scale 2) when they were planted in the control soil (data not shown).

To determine stability of resistance to *Fusarium* in the most resistant somaclones selected from the first cycle, we maintained 54 somaclones with a disease rating of 2 or less in the greenhouse for further disease screening. Of 54 somaclones maintained in the greenhouse, 15 (27.7%) developed spears that could be used as explants for micropropagation. The 15 somaclones were then vegetatively micropropagated as described above. Only two somaclones (R7 and R4) developed healthy roots in vitro. At least 12 micropropagated plants each of R7 and R4 were rescreened with FOA50 in the greenhouse by using a randomized complete block design. Somaclonal line R7 was highly significantly ($P < 0.01$) more resistant to FOA50 than were the vegetatively micropropagated Lucullus 234 parental plants. Of 12 micropropagated plants of R7, 91.7% were rated 1 or 2, in contrast to 6.67% of the parental plants (Table 2). Somaclonal line R4 had highly significantly ($P < 0.01$) more resistance to FOA50 than did the vegetatively micropropagated Lucullus 234 parental plants. Of 18 micropropagated plants of R4, 50% of them were rated at 1 or 2, in contrast to 6.7% of the parental plants (Table 3). These results indicated that the increased levels of resistance of R7 and R4 to the isolates of *Fusarium* spp. from the first cycle of screening were stable after vegetative micropropagation. In addition, no morphological and growth differences between these lines and the parental cultivar were observed.

Although Lucullus 234 was more resistant to FOA10 and FM12 in comparison with 90 other cultivars and breeding lines of *A. officinalis* tested (39), our results showed that the vegetatively micropropagated plants of Lucullus 234 seed plants were as susceptible to FOA50 as UC 157 (Tables 2 and 3).

Asparagus somaclones of Lucullus 234 expressed a higher level of resistance to the isolates of *Fusarium* spp. than did the vegetatively micropropagated Lucullus 234 parental plants after the first cycle of screening. The vegetatively micropropagated plants of two somaclones with higher resistance levels were rescreened to the most virulent isolate FOA50. These two somaclonal lines, R7 and R4, were highly significantly more resistant than the parental plants, indicating the maintenance of the increased resistance in R7 and R4 lines after vegetative micropropagation. More highly resistant somaclones were regenerated at a high frequency of 24.2% (vs. 8.3% in the parental plants of Lucullus 234) from the moderately resistant cultivar Lucullus 234 after the first cycle of screening. Ninety-two percent of the vegetatively micropropagated plants of R7 had disease ratings of 1 or 2, whereas

Table 1. Disease rating of asparagus plants screened with *Fusarium oxysporum* f. sp. *asparagi* (isolates FOA10 and FOA50) and *F. proliferatum* (isolates FM12 and FM49) in the greenhouse

Plant source	No. of plants in disease rating scales[y]					Mean disease rating[z]
	1	2	3	4	5	
UC 157 (susceptible control)	0	21	49	33	17	3.38 A
Micropropagated seed plants of Lucullus 234	10	50	31	13	16	2.74 B
Somaclones of Lucullus 234	29	49	19	10	13	2.41 C
Sprengeri (resistant control)	61	57	2	0	0	1.51 D

[y] One hundred twenty plants of each plant source were screened at four different times. Disease was assessed using a visual rating scale: 1 = no disease, 2 = less than 25% diseased, 3 = less than 50% diseased, 4 = less than 75% diseased, 5 = greater than 75% diseased.

[z] Means of disease rating within a column with different letters are significantly different (Duncan's multiple range test, $P < 0.05$).

93.3% of the parental plants had rating of 3 or greater. Fifty percent of the vegetatively micropropagated plants of R4 had a rating of 1 or 2, while 93.3% of the parental plants had a rating of 3 or more.

Somaclonal variation could be affecting the resistance to *Fusarium* by a mutation resulting in the addition of another resistant gene or by amplification of a naturally existing resistant gene in Lucullus 234, although the resistant gene in Lucullus 234 has not been determined. The resistant somaclonal lines R7 and R4 were found to have a normal diploid (2n = 20), as did the parental Lucullus 234 cultivar, and to have carried DNA sequence variations that were unique to the parental cultivar (6) and to its somaclones susceptible to *F. o.* f. sp. *asparagi* (Y. H. Dan and C. T. Stephens, *unpublished*). It is not clear whether the resistance to the *Fusarium* spp. in R7 and R4 is due to monogenic or multigenic mutations.

Resistance to *F. oxysporum* is found in 15 different vegetable crops, and over half of these are controlled by a single dominant gene (18). Somaclones derived from protoplasts of tomato were resistant to *F. o.* f. sp. *lycopersici* race 2, and this resistance was due to a single dominant gene mutation (35). Evans and Sharp (15) recovered 13 single-gene mutations from 230 regenerated tomato plants from leaf explants. A tomato variety (DNAP 17) developed from the 230 regenerated plants expressed the resistance to *F. o.* f. sp. *lycopersici* race 2 and had good agronomic performance. In this case, the resistance was the result of a single-gene, dominant mutation (14). The mode and nature of resistance to *F. proliferatum* was barely reported. Tolerance, such as related to plant vigor, seems to be a mechanism against *F. proliferatum* (5).

In further experiments, the resistant somaclonal lines, R7 and R4, should be tested in the field for resistance to *Fusarium* spp. or to other biotic factors implicated in asparagus decline, such as asparagus virus I and II (49) and *Stemphylium vesicarium* (42), after vegetative micropropagation or sexual propagation by crossing with other horticulturally desirable cultivars of asparagus. These resistant somaclonal lines could provide a source of increased resistance to *F. oxysporum* and *F. proliferatum* in an asparagus breeding program.

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Table 2. Disease rating of vegetatively micropropagated plants of the more resistant somaclone R7 rescreened with *Fusarium oxysporum* f. sp. *asparagi* (isolate FOA50) in the greenhouse

Plant source	No. of plants in disease rating scales[y]					Mean disease rating[z]
	1	2	3	4	5	
UC 157 (susceptible control)	0	1	9	2	3	3.47 A
Micropropagated seed plants of Lucullus 234	0	1	12	1	1	3.13 A
Micropropagated plants of somaclone R7 of Lucullus 234	3	8	1	0	0	1.83 B
Sprengeri (resistant control)	14	1	0	0	0	1.07 C

[y] Twelve vegetatively micropropagated plants of R7 were screened with FOA50. Disease was assessed using a visual rating scale: 1 = no disease, 2 = less than 25% diseased, 3 = less than 50% diseased, 4 = less than 75% diseased, and 5 = greater than 75% diseased.

[z] Means within a column with different letters are significantly different (Student's t test, $P < 0.01$).

Table 3. Disease rating of vegetatively micropropagated plants of the more resistant somaclone R4 rescreened with *Fusarium oxysporum* f. sp. *asparagi* (isolate FOA50) in the greenhouse

Plant source	No. of plants in disease rating scales[y]					Mean disease rating[z]
	1	2	3	4	5	
UC 157 (susceptible control)	0	1	5	5	4	3.80 A
Micropropagated seed plants of Lucullus 234	0	1	7	4	3	3.60 A
Micropropagated plants of somaclone R4 of Lucullus 234	1	8	6	2	1	2.67 B
Sprengeri (resistant control)	14	1	0	0	0	1.07 C

[y] Eighteen vegetatively micropropagated plants of R4 were screened with FOA50. Disease was assessed using a visual rating scale: 1 = no disease, 2 = less than 25% diseased, 3 = less than 50% diseased, 4 = less than 75% diseased, and 5 = greater than 75% diseased.

[z] Means within a column with different letters are significantly different (Student's t test, $P < 0.01$).

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