ASSESSMENT OF A METHOD USING PLANTLETS GROWN PREVIOUSLY *IN VITRO* FOR STUDYING RESISTANCE OF *PRUNUS CERASIFERA* EHR. (MYROBALAN PLUM) TO *MELOIDOGYNE* SPP.

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ABSTRACT

Esmenjaud D., J. C. Minot, R. Voisin, G. Salesses, R. Poupet, and J. P. Onesto. 1993. Assessment of a method using plantlets grown previously *in vitro* for studying resistance of *Prunus cerasifera* Ehr. (myrobalan plum) to *Meloidogyne* spp. Nematropica 23:41–48.

Plantlets of two genotypes of *Prunus cerasifera* resistant to *Meloidogyne arenaria*, and one susceptible genotype, were rooted *in vitro* with and without 0.5 ppm indolebutyric acid (IBA). Seventy days after transplanting plantlets into greenhouse pots containing a sandy soil, they were inoculated with 0, 500, 1 500, or 4 500 second-stage juveniles (J2) of *M. arenaria*. Two months later, root galling and nematode development were evaluated. Galling was absent in the resistant genotypes P.1079 and P.2175 at all inoculum levels and no nematode reproduced regardless of the *in vitro* rooting medium used. In IBA-rooted plantlets of the susceptible genotype P.2032, nematodes reproduced normally and the ratios of developmental stages were unaffected by inoculum level, suggesting no appreciable intraspecific competition even at 4 500 J2/plant. Plantlets of P.2032 that had been rooted in medium supplemented with IBA were much more homogeneous than those rooted without IBA. The results indicate, therefore, that *in vitro* rooting in IBA-supplemented medium, followed by inoculation with 1 500 to over 4 500 J2/plant, can be used to study resistance of *P. cerasifera* to *M. arenaria* and presumably to other *Meloidogyne* spp.

Key words: hormone, indolebutyric acid, *in vitro* propagation, *Meloidogyne arenaria*, plum, *Prunus cerasifera*, resistance.

RESUMEN

Esmenjaud D., J. C. Minot, R. Voisin, G. Salesses, R. Poupet y J. P. Onesto. 1993. Evaluación de un método usando plántulas propagadas previamente *in vitro* para estudiar la resistencia de *Prunus cerasifera* Ehr. (ciruelo myrobalan) frente a *Meloidogyne* spp. Nematrópica 23:41–48.

Plántulas de tres genotipos de *Prunus cerasifera*, dos resistentes y uno susceptible a *Meloidogyne arenaria*, se enraízaron *in vitro* con y sin 0.5 ppm de ácido indolbutírico (AIB). Setenta días después del transplante a un suelo arenoso se inocularon con 0, 500, 1 500 o 4 500 segundos estadíos juveniles (J2) de *M. arenaria*. Dos meses más tarde, se evaluó el agallamiento de las raíces y la reproducción del nematodo. No se encontraron agallas en los genotipos resistentes (P.1079 y P.2075) en ningunos de los niveles de inóculo. El nematodo no se reprodujo independientemente de la clase de medio *in vitro* usado para enraízar las plántulas. En plántulas del genotipo susceptible (P.2032) enraízadas con AIB, *M. arenaria* se reprodujo normalmente y los proporciones entre los estadíos de desarrollo no fueron afectados por el nivel de inóculo, lo cual sugiere una ausencia de competencia interespecífica notable aún a los 4 500 J2/planta. Las plántulas de P.2032 enraízadas en medio conteniendo AIB fueron mucho más homogéneas que las enraízadas sin AIB. En consecuencia, los resultados indican que la técnica de enraízar plántulas *in vitro* en un medio con AIB, sequido por la inoculación en macetas con 1 500-4 500 J2/planta, puede ser utilizado en estudios para determinar la resistencia de *P. cerasifera* frente a *M. arenaria*, como también a otras especies de *Meloidogyne*.

Palabras clave: ácido indolbutírico, ciruelo, hormona, *Meloidogyne arenaria*, propagación *in vitro*, *Prunus cerasifera*, resistencia.

INTRODUCTION

Meloidogyne arenaria (Neal) Chitwood, *M. incognita* (Kofoid & White) Chitwood, *M. javanica* (Treub) Chitwood and *M. hispanica* Hirschmann reproduce readily on *Prunus* spp. The three first species have been reported on stone fruit crops in many countries but the fourth species has been recently described (5) and seems to be limited to southern Spain (Scotto La Massèse, personal communication). In France, *M. arenaria* appears to be the most common root-knot nematode found in fruit tree orchards (11).

As a source of resistance to root-knot nematodes, *P. cerasifera* Ehr. (myrobalan plum) seems promising (11). Many genotypes in this species present desirable agronomic characteristics such as graft compatibility with peach and almond, wide adaptation to different soils (especially finely textured, waterlogged, and calcareous soils) and a lower susceptibility than peach to *Verticillium* wilt, crown gall, and crown rot (9).

The host response in *P. cerasifera* genotypes to *M. arenaria* ranges from susceptible to highly resistant (4). Results of a recent study utilizing plants derived from hardwood cuttings suggest that root-knot nematode resistance is controlled by at least one major dominant gene (11). Further studies are needed on such biological characteristics of resistance as host-parasite specificity, penetration and emigration of juveniles, and interactions with other pathogens (*e.g. Pratylenchus* spp. and *Agrobacterium tumefaciens*).

This paper deals with the assessment of a method for evaluating resistance using plantlets grown *in vitro*. Plantlets can be easily propagated *in vitro* throughout the year and are much more homogeneous than cuttings. As reported by several authors (8,3,6), a modification of resistance can be induced by exogenous hormones. The first purpose of this research was to ensure that the expression of resistance by plants grown in greenhouse pots is not modified by the presence of exogenous hormones in the previous *in vitro* rooting medium . A second objective was to identify a suitable level of inoculum for use in future studies, high enough to readily detect differences in resistance yet low enough to prevent appreciable intraspecific competition between nematodes.

MATERIALS AND METHODS

Three Myrobalan plum genotypes were chosen. Two are resistant to the Monteux isolate of *M. arenaria*. These are P.1079, which possesses in the homozygous state at least one major dominant gene of resistance, and P.2175, which is believed to have the same major gene(s) in the heterozygous state (11). The third genotype, P.2032 (Myrabi), is susceptible to the same nematode isolate.

Propagation and rooting of plantlets: All genotypes were propagated on Murashige and Skoog medium (10) supplemented on a 1-L basis with 30 gram saccharose, 0.4 mg thiamine HCl, 1 mg vitamin B, 100 mg myoinositol, 200 mg sequestrene^{R}, 0.1 mg gibberellic acid (GA), 1 mg 6-benzylamino purine (BAP), and 0.05 mg of indolebutyric acid (IBA). One month later, propagated shoots were rooted on the same medium without any growth regulator (no GA, BAP, or IBA) (denoted hormoneless or HL plantlets) or were rooted on the same medium containing only 0.5 mg IBA (= 0.5 ppm) (denoted IBA plantlets). All culture media were adjusted to pH 5.3 before sterilizing. Plantlets were maintained at 22 °C with a

16-hr photoperiod provided by cool-white fluorescent lamps during propagation and rooting. After 1 month, plantlets were transplanted into 1: 1 (v:v) fine sand plus perlite substrate within tanks (50 × 30 × 15 cm) in an acclimatization chamber. Plantlets were acclimatized at 20–22 °C for 2 weeks, and transferred into the greenhouse for 2 more weeks, then transplanted into 0.25-L individual containers filled with a sterilized 4:1 mixture (v:v) of fine sand and loamy soil. A sand particle size of 0.1–0.5 mm was used based on the results of Wallace (12). Containers were drip irrigated daily with a commercial nutrient solution for ligneous plantlets (Algoflash, Algochimie, Tours, France) at pH 5.8 and maintained in the greenhouse from April to July at 25 ± 3 °C. Eight hardwood cuttings of each cultivar rooted during the previous year were used as controls. Hardwood cuttings were planted in 5-L containers and inoculated after 2 months with one galled tomato according to the method described by Esmenjaud *et al.* (4).

Inoculation with nematodes: Second-stage juveniles (J2) were obtained in a mist chamber from tomato roots previously inoculated with the Monteux isolate of *M. arenaria*. The isolate had been reared from a single egg mass culture on tomato cv. St Pierre. Seventy days after transplanting into pots (from *in vitro* culture), each set of plantlets (HL and IBA) was inoculated with 0, 500, 1 500 and 4 500 J2/plantlet. Freshly hatched juveniles (24–48 hr old) were deposited into four 2-cm-deep holes 1 cm from the base of the stem. Each combination of inoculum level and hormone treatment was replicated 17 times for P.1079 and 16 times for P.2175. Sufficient plantlets for 15 replicates of P.2032 at each inoculum level and hormone treatment were propagated but many of the HL plantlets were considered excessively heterogeneous. These were discarded, leaving enough HL P.2032 plantlets for only eight replicates at each inoculum level that were compared with eight replicates of IBA plantlets at each inoculum level. The mean initial height of the 15 replicates of IBA plantlets was 50% higher than that of the HL plantlets.

Criteria for comparison: On the date of inoculation, the initial shoot height of the plantlets was measured. Two months after inoculation, plantlets were harvested and final shoot height, root weight, and top weight measured. Each plantlet was given a 0–5 root-knot index according to the following scale derived from Barker (1): 0 = 0%; 1 = 1–10%; 2 = 11–30%; 3 = 31–70%; 4 = 71–90%; 5 = more than 90% of the root system galled. To extract nematodes from roots, roots were macerated in water using an ultra-grinder (20 000 rpm) for 2 s, then passed through a 250-µm-pore sieve into a beaker; intact root pieces retained on the sieve were ground again. Nematodes collected in the beaker were separated from root debris by centrifugal flotation (7). Females, males, J3–J4, J2, and eggs were counted under a stereomicroscope. Plant heights, relative growth (final/initial heights), and weights were analyzed by a one-way analysis of variance. Means were compared by Newman-Keuls multiple range test ($P \leq 0.05$). The nematode parameters analyzed included gall index and the numbers of females, males, J3–J4, eggs, the total of [females + males + J3–J4] and the total of [J2 + eggs] per plant as well as per 10 g of roots. Gall index ratings and $\log_{10}(X + 1)$ transformed numbers of nematodes for each genotype were separately submitted to a factorial analysis of variance with two factors (hormone treatment and level of inoculum). Means were compared

by Newman-Keuls multiple range test ($P \leq 0.05$).

RESULTS

Effect of IBA on plant growth and nematode development: There was no significant difference between plant development of the IBA and HL plantlets of either resistant genotype (Table 1). Galling and nematodes of all developmental stages at harvest were absent on all *in vitro* propagated plantlets of both genotypes, as well as on the hardwood cutting controls. HL plantlets of the susceptible genotype P.2032 were significantly smaller than IBA plantlets (Table 2); however, there was no significant effect of IBA on any parameter of nematode development (Table 3). Results expressed in nematode numbers per gram of analyzed roots (not reported here) were similar.

Effect of the level of J2 inoculum: No significant differences related to inoculum level were observed for any plant or nematode parameter in either of the resistant genotypes P.1079 and P.2175 (Table 1). Statistical analysis of data for the susceptible P.2032 plantlets indicated no significant effect of inoculum on any plant parameter (Table 2) and few effects on nematode parameters (Table 3). However, HL plantlets were much more heterogeneous than IBA plantlets with coefficients of variation for most parameters several times those of the IBA plantlets. Since the analysis assumed homogeneity of variance for each parameter, a condition not met by the data set, the data for the 15 replicates of the IBA treated plantlets were re-analyzed separately from the HL plantlet data (Table 4). Still no difference in plant growth was observed (data not shown). However, the gall index and numbers of females, J2, and eggs differed significantly at all inoculum levels, increasing more or less proportionately to the amount of inoculum applied. Although the numbers of J3–J4 and males when considered separately, and the total of all post–J2 stages did not differ between the 500 and 1 500 inoculum levels, they did differ between the 1 500 and the 4 500 levels. Hardwood cutting controls had a gall index of 2.4, which was equivalent to the highest level in IBA plantlets.

DISCUSSION

In our study, rooting of plantlets in Murashige and Skoog medium supplemented with 0.5 ppm of the hormone IBA had no marked effect on the growth of the two resistant genotypes. However, an obvious effect was observed in the susceptible genotype P.2032, namely that larger plantlets with greater homogeneity due to more uniform *in vitro* rooting could be obtained with IBA. This hormone did not decrease the resistance of resistant genotypes detectably. As for the susceptible genotype P.2032, in most cases the mean number of nematodes of each developmental stage recovered from roots were higher on IBA than on HL plantlets and high nematode counts were generally associated with greater than average root weights, but these differences were small compared with differences in nematode populations due to inoculum level and were not statistically significant. Therefore, from the standpoint of overall nematode reproduction, IBA treatment had little if any effect on the expression of resistance or susceptibility. As expected, the hormonal modification of resistance observed by Kochba and Samish (8) for peach plantlets grown in medium containing from 0.1 ppm 1-naphtylacetic acid was not obtained for myrobalan plantlets that had been exposed to 0.5 ppm IBA, 70 days earlier. Actually, the initial exo-

Table 1. Effect of previous rooting in a medium containing 0.5 ppm of indolebutyric acid on plant growth and nematode development in the resistant genotypes P.1079 and P.2175 of *Prunus cerasifera* grown in a sandy soil and inoculated with three levels of *Meloidogyne arenaria* juveniles (J2). Gall index of the hardwood-cutting controls (eight replicates) were null for the two genotypes.

Genotype	Inoculum level (J2/plant)	Hormone treatment	Initial height (cm)	Final height (cm)	Fresh root weight (g)	Fresh top weight (g)	Gall index (0–5)	Juveniles per plant
P.1079	0	IBA[z]	9.8	17.4	2.9	2.2	0	0
		HL	9.2	15.7	2.8	2.2	0	0
	500	IBA	9.8	15.1	2.9	2.1	0	0
		HL	8.7	14.7	2.3	2.0	0	0
	1 500	IBA	9.7	16.5	2.9	2.4	0	0
		HL	10.2	14.5	2.8	2.0	0	0
	4 500	IBA	10.2	14.5	2.8	2.0	0	0
		HL	9.3	17.2	2.5	1.9	0	0
P.2175	0	IBA	6.3	10.9	2.0	2.3	0	0
		HL	6.2	12.4	2.5	2.3	0	0
	500	IBA	5.7	9.7	2.3	1.6	0	0
		HL	6.1	10.2	2.5	1.9	0	0
	1 500	IBA	5.7	10.9	2.9	2.2	0	0
		HL	6.1	11.5	2.4	2.1	0	0
	4 500	IBA	6.0	8.1	2.1	1.6	0	0
		HL	6.3	11.0	2.5	2.3	0	0

Data are means of 17 (P.1079) and 16 (P.2175) replicates.
[z]IBA = indolebutyric acid; HL = hormoneless medium.

Table 2. Effect of previous rooting in a medium containing 0.5 ppm of indolebutyric acid on plant growth in the susceptible genotype P.2032 of *Prunus cerasifera* grown in fine sand and inoculated with four levels of *Meloidogyne arenaria* juveniles (J2). Gall index of the hardwood-cutting control (eight replicates) was 2.4.

Hormone treatment	Inoculum level (J2/plant)	Initial height (cm)	Final height (cm)	Fresh root weight (g)	Fresh top weight (g)
IBA[x]	0	6.7	12.7	2.6	1.9
	500	6.7	12.4	2.5	1.8
	1 500	6.8	12.9	2.6	2.0
	4 500	6.9	12.0	2.7	1.9
		NS[y]	NS	NS	NS
HL	0	4.5	9.2	1.8	1.4
	500	4.8	11.1	1.9	1.4
	1 500	4.6	9.5	2.3	1.5
	4 500	4.4	9.8	2.2	1.4
		NS	NS	NS	NS
Mean[z]	IBA	6.8 a	12.5 a	2.6 a	1.9 a
	HL	4.5 b	9.9 b	2.1 b	1.4 b

Data are means of eight replicates.
[x]IBA = indolebutyric acid; HL = hormoneless medium.
[y]NS = Non significant ($P \leq 0.05$).
[z]Means of the four inoculum levels. Means in columns followed by the same letter do not differ according to the Newman-Keuls multiple range test ($P \leq 0.05$).

genous hormone probably is metabolized and substituted by endogenous hormones produced at a normal level by the plant itself.

No reduction in plant growth due to inoculation with nematodes was measured for any of the three genotypes. This suggests that young plants of the susceptible genotype possess an appreciable level of tolerance to nematode infection. However, rates of penetration estimated by the ratio [females + males + J3–J4]/inoculum, were only 6.5%, 3.2%, and 2%, respectively, for the 500, 1 500, and 4 500 J2/plant inoculum levels. These very low ratios indicate that either the density of roots was not high enough to allow the juveniles to find and penetrate the roots, or P.2032 possesses a partially effective resistance mechanism that limits tissue penetration and (or) the establishment of feeding sites by J2. In any event, there probably was little if any intraspecific competition. This hypothesis is supported by similar sex ratios and similar ratios among developmental stages observed at all inoculum levels. Further studies, such as those of Burdett *et al.* (2) on *P. persica* Batch, are needed to better understand infection and development by *M. arenaria* on *P. cerasifera*.

In conclusion, the best conditions for obtaining homogeneous plantlets of *P. cerasifera* for use in root-knot nematode resistance studies without modifying the resistance parameters were obtained by rooting plantlets in *in vitro* culture rooting medium supplemented with 0.5 ppm IBA, 70 days prior to inoculation with J2. The best levels of inoculum to apply to the plantlets would depend on the aim of the experiment. Where it is desired to distinguish levels of resistance or susceptibility with minimal intraspecific competition between nematodes, a range from 1 500 to more than 4 500 J2 would be suitable.

Table 3. Effect of previous rooting in a medium containing 0.5 ppm indolebutyric acid on nematode development in the susceptible genotype P.2032 of *Prunus cerasifera* grown in a sandy soil and inoculated with three levels of *Meloidogyne arenaria* juveniles (J2).

Hormone treatment	Inoculum level (J2/plant)	Gall index (0–5)	Eggs	J2	J3–J4	Males	Females	J2 + eggs	Females + males + J3–J4
IBA[v]	0	0	0	0	0	0	0	0	0
	500	1.5	93	73	3	13	16	166	32
	1 500	1.8	169	144	2	18	22	313	42
	4 500	2.3	232	195	9	42	44	427	95
HL	0	0	0	0	0	0	0	0	0
	500	1.5	89	60	2	12	13	149	23
	1 500	1.7	147	98	11	23	30	245	63
	4 500	1.8	180	174	5	14	29	354	48
Mean[x]	0	0 a[w]	0 a	0 a	0 a	0 a	0 a	0 a	0 a
	500	1.5 b	91 b	66 b	2 b	12 b	14 b	157 b	27 b
	1 500	1.7 bc	158 b	121 b	6 bc	20 b	26 c	279 b	52 c
	4 500	2.0 cd	206 b	184 b	7 c	28 b	36 c	390 b	71 c
Mean[y]	IBA	1.9	164	137	5	24	27	302	56
	HL	1.7	138	110	6	16	24	248	46
		NS[z]	NS	NS	NS	NS	NS	NS	NS

Data are means of eight replicates.

[v]IBA = indolebutyric acid; HL = hormoneless medium.

[w]Non transformed means are pesented but data were transformed to $\log_{10}(X+1)$ for statistical analysis (except gall index). Means in columns followed by the same letter do not differ according to Newman-Keuls multiple range test ($P \leq 0.05$).

[x]Means of the two hormone treatments.

[y]Means of the three inoculum levels.

[z]NS = Non significant ($P \leq 0.05$).

Table 4. Development of *Meloidogyne arenaria* under greenhouse conditions in the susceptible genotype P.2032 of *Prunus cerasifera*, 2 months after inoculation with 500, 1 500, and 4 500 second-stage juveniles (J2) per plant. Plantlets were propagated *in vitro* and rooted in medium containing 0.5 ppm indolebutyric acid, 70 days before inoculation.

Inoculum level (J2/plant)	Gall index (0–5)	Eggs	J2	J3–J4	Males	Females	J2 + eggs	Females + males + J3–J4
500	1.5 a[z]	90 a	85 a	3 a	14 a	15 a	175 a	33 a
1 500	1.9 b	209 b	166 b	3 a	23 a	22 b	375 b	48 a
4 500	2.3 c	361 c	199 c	7 b	37 b	45 c	560 c	89 b

Data are means of 15 replicates. Non transformed data are presented but data were $\log_{10}(X+1)$ transformed for statistical analysis (except gall index).

[z]Means in columns followed by the same letter do not differ according to Newman-Keuls multiple range test ($P \leq 0.05$).

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