Evaluation of Castor Bean Resistance to Sclerotial Wilt Disease Caused by *Macrophomina phaseolina*

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ABSTRACT

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Macrophomina phaseolina was found to be the causal agent involved in a wilting disease of castor bean *(Ricinus communis)* in South Africa, Costa Rica, and Brazil. Resistance to *M. phaseolina* was found in a small-seeded *Ricinus persicus* and incorporated in cultivated lines. Four different methods were evaluated for resistance screening. Both field and inoculated glasshouse trials demonstrated that resistance was expressed from seedling stage to seed set. This is the first report on castor bean resistance to *M. phaseolina*.

Castor bean, *Ricinus communis* L., is an important oilseed produced in different countries throughout the world. The oil is used for industrial purposes and is considered strategic by many governments (8). Total world production has remained stable over the last 25 years and has exceeded one million tons per year since the 1980s (8).

The most important castor bean pathogens are soilborne fungi. They attack the roots and the basal portion of the stem from the seedling to the harvest stage. Fusarium wilt caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ricini* (Wr.) Gordon and sclerotial wilt caused by *Macrophomina phaseolina* (Tassi) Goidanich are considered to be among the most serious diseases (23). The importance of these two fungi varies among countries. Long dry spells with high temperatures generally favor sclerotial wilt (10). Chemical control of these fungi is very difficult and neither profitable nor advisable. Cultural practices and use of resistant cultivars can be combined to substantially reduce losses from these diseases in some susceptible crops (10,30). Castor bean genetic resistance against Fusarium wilt caused by *F. oxysporum* f. sp. *ricini* has been characterized (23), but resistance against *M. phaseolina* has never been described in the literature.

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Elf-Atochem's seed division recently characterized castor bean germ plasm resistant to a seedling stage wilt under field conditions. This resistance, found in a small-seeded *Ricinus persicus* G. Pop., was incorporated in cultivated lines by backcrossing. The resistance was effective in warm and dry locations in South Africa, Costa Rica, and Brazil. The objective of this research was to evaluate castor bean resistance to sclerotial wilt by means of several screening methods under greenhouse and laboratory conditions.

MATERIALS AND METHODS

Isolates of *Macrophomina phaseolina*. Isolations were performed on young wilted plants, 28 to 60 days after emergence, at several locations in nurseries and seed production fields in 1991 in Costa Rica and in 1992 in Brazil (Bahia-Nordeste).

Tissues excised from small, lateral, dying roots, necrotic taproots, and the margin of black spots on basal stems were washed, surface disinfested with 1% sodium hypochlorite for 1 to 3 min, rinsed twice in sterile water, and blotted dry with sterile paper. Excised tissues were incubated on one of the following three media: potato dextrose agar (PDA) acidified with 25% lactic acid and supplemented with 50 µl of streptomycin sulfate per ml; malt agar supplemented with 100 µg of chloramphenicol per ml; and Komada's medium, selective for *Fusarium* spp. (19). Culture plates were incubated in the dark at 25°C and hyphal tips from margins of resulting colonies were transferred to PDA for identification and study. A French isolate of *M. phaseolina* (AJF), isolated on sunflower, also was used for host pathogenicity studies.

Plant genotypes. The field-susceptible genotype N382 came from a cross with USDA line US 3-415-9 (interspersed) and a waxy-green line of South African origin. Resistant genotypes resulted from crosses between an indigenous small-seeded *Persicus* sp. found in Madagascar and the KL6 line. With one backcross to KL6, single plants were then crossed with C6E (Elf-Atochem line). The two resistant lines used in this study (N7 and C8), were selected from this cross under naturally infested conditions.

Virulence and pathogenicity tests: Soil inoculation method. *M. phaseolina* isolates were cultured on a maize/sand medium containing 1 liter of dry sand, 160 ml of maize meal, and 150 ml of water. Five hundred grams of this medium was poured into 4-liter plastic bags closed with a cellulose cork and autoclaved for 2 h at 120°C. Each bag was then inoculated with 10 mycelial plugs (8 mm in diameter) cut from the margin of a 4-day-old colony growing on PDA. The culture was harvested 15 days after inoculation and dried 48 h at room temperature under a laminar flow hood. Propagule concentration was determined by plating the inoculum on PDA. This inoculum was then directly used or stored at 4°C in sterile plastic boxes containing silica gel as a desiccant (10 g of silica gel for 100 g of dried inoculum).

Soil was infested by mixing the inoculum with a mixture of compost (Orga-fleur, Fertilaquitaine, Landiras, France) and sterilized sand (1/3, vol/vol). The infested soil was poured into 50-ml plastic tubes (25330-50, Centrifuge Tube, Corning, NY) having a 4-mm hole and a plug of cotton at the base of each tube.

Surface sterilized (2.5% sodium hypochlorite for 5 min) castor bean seeds were sown in vermiculite. The germination trays were incubated at 28°C in a growth chamber with a 16-h photoperiod and a light

intensity of 250 μE s^{-1} m^{-2}. One germinated seed (2 to 3 days old) was then planted 2 cm below the infested potting mix surface in each tube and incubated under the same environmental conditions. Tubes were placed on a stand in a plastic tray containing a thin layer of water kept at a constant level (about 1 cm).

Disease incidence was evaluated by recording every 2 days the number of live plants (presence of green apex) and the weight of each plant 21 days after transplanting.

Control plants were sown in noninfested soil and maintained as above.

Blotting-paper inoculation method. Four-day-old seedlings, grown under conditions described previously, were carefully uprooted and washed free of vermiculite before inoculation. Inoculum consisted of mycelial plugs (6 mm in diameter) cut from the margin of a 4-day-old colony grown on PDA in the dark at 28°C. One mycelial plug was placed in the middle of the taproot of each seedling. The seedlings were then placed between two sheets of blotting paper (8 cm wide × 11 cm long) so that the root system was covered while the leaves remained exposed. The upper part of the blotting paper was rolled around a small glass rod (12 cm long, 5 mm in diameter) and the two blotting paper sheets were then stapled. The seedlings were suspended with the glass rods over an open box (40 cm long × 10 cm wide × 12 cm deep) containing a 2-cm layer of water. Seedlings were incubated in a growth chamber under the conditions previously described. Control plants were treated with noninoculated PDA. Ten to 15 seedlings were inoculated to evaluate the virulence of fungal isolates and resistance of castor bean genotypes. Fifteen days after inoculation, individual seedlings were scored for the extent of root infection (length of root lesion).

Stem tape inoculation method. Plants were cultured under greenhouse conditions at an average temperature of 28°C. Plants were irrigated with 0.5 g of Mairol nutrient solution (Mairol 14:12:14, Schering agrochimie, Rungis, France) per liter. Plant hypocotyls were superficially wounded by peeling the epidermis with a razor blade, 2 cm above the soil surface. The wounded area was about 1 mm deep and 5 mm long. Fungi were grown on PDA at 28°C. A mycelial plug, 4 mm in diameter, harvested at the periphery of a growing 4-day-old colony, was placed against the wound and covered with cellophane tape. Control plants were wounded and inoculated with a sterile PDA plug. Disease level was recorded by assessing the percentage of dead plants or the length of stem lesion.

Toothpick inoculation method. Plants were cultured in the greenhouse under conditions described above (stem tape method). Inoculum of *M. phaseolina* was produced on sterile toothpicks. Toothpicks were boiled in water for 1 h, air dried, and loosely packed in glass jars containing potato dextrose broth covering the lower one-third of the toothpicks. Jars were autoclaved twice for 20 min at 120°C. When cooled, the toothpicks were plated on PDA containing a 4-day-old *M. phaseolina* culture and incubated in darkness at 28°C. Ten days later, plants were inoculated by inserting a toothpick in the middle of the first internode. Control plants were inoculated with noninfested toothpicks. Disease reaction was scored by measurement of plant survival and/or the length of stem lesion.

Data analysis. Virulence trials of *M. Phaseolina* strains consisted of completely randomized trials with four replicates of nine plants. Pathogenicity comparison experiments of Brazilian (no. 199) and French (AJF) isolates and resistance evaluation of susceptible (382I) and resistant (C8, N7) lines were performed in randomized trials; each plant was considered to be an experimental unit. All experiments were repeated at least once with similar results. Data were analyzed by means of the computer program STATITCF (ITCF Institute, Boigneville, France).

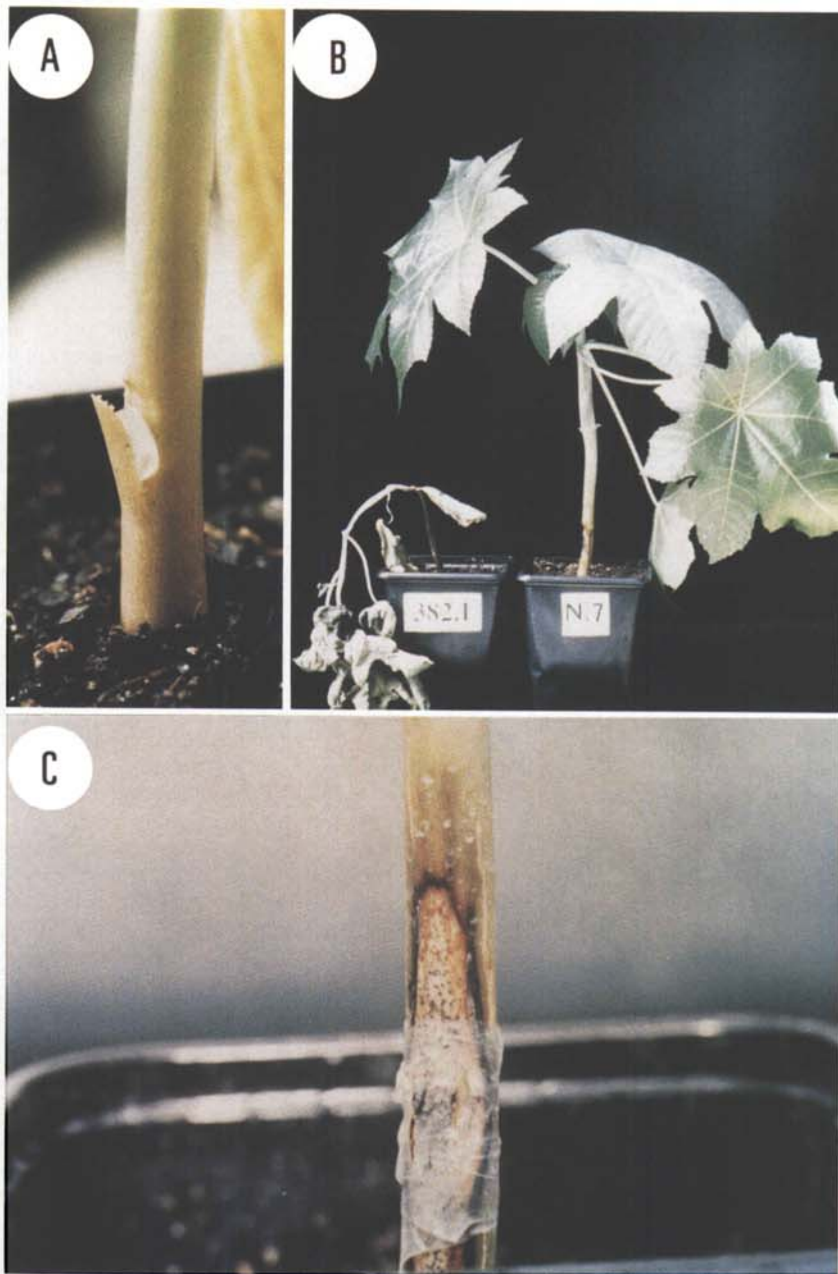

Fig. 1. Stem-tape inoculation method. **(A)** Plant hypocotyls were superficially wounded and a mycelial plug was placed against the wound. **(B)** Thirty-one-day-old plants were inoculated with *M. phaseolina*. After 15 days, a large lesion could be noted on the stem of the susceptible genotype (382I). On the resistant genotype (N7) only a limited lesion (half the length of the susceptible genotype) could be noted. The large lesion on the susceptible castor bean genotype often induced plant wilting. **(C)** Twenty-one days after inoculation with *M. phaseolina* Brazilian isolate no. 199, pycnidia were observed on the stem lesion.

Table 1. Virulence of *Macrophomina phaseolina* isolates on castor bean, resulting from the stem tape inoculation method

Strain no.	Plant death (%)[x]	
	5 days[y]	10 days
23	0 a[z]	0 a[z]
59	0 a	0 a
146	0 a	22.2 b
167	22.2 b	55.5 b
197	44.4 b	88.8 c
199	100 c	100 c
166	100 c	100 c

[x] Isolate virulence analysis carried out on four replicates of nine plants of the susceptible genotype, 382I.
[y] Days after inoculation.
[z] Means followed by the same letter are not significantly different at $P = 0.05$, using the Newman-Keul's test.

Table 2. Determination of lesion length on seven plant species after stem tape artificial inoculation with *M. phaseolina* isolates originating from Brazil and France, collected on castor bean and sunflower, respectively

Plant species	Cultivar	Age (day)[w]	Plant no.[x]	*M. phaseolina* isolate	
				Brazilian (no. 199)[y]	French (AJF)[y]
Castor bean	382I	22	10	8.54 ± 1.52	6.87 ± 1.23
Sunflower	Albena	18	24	1.88 ± 0.12	1.46 ± 0.16
Soybean	Chandor	13	18	1.62 ± 0.12	1.21 ± 0.15
Maize	DK524	13	20	0	0
Pea	Douce Provence	13	24	5.8 ± 0.98	4.44 ± 1.93 *[z]
Sorghum	Ambre	27	25	7.50 ± 0.98	2.52 ± 0.5 *
French bean	Nain cocktel	20	20	5.19 ± 0.99	1.40 ± 1.01 *

[w] Plant age at inoculation time.
[x] Number of plants inoculated for pathogenicity analysis.
[y] Values are mean lesion length (± SD) assessed 8 days after inoculation.
[z] Indicates significant difference between Brazilian (no. 199) and French (AJF) isolates of *M. phaseolina* based on Student's *t* test ($P = 0.05$).

Differences between isolates or genotypes in the tests were analyzed by using Student's *t* test or one-way analysis of variance, followed by mean separation using the Newman-Keul's test.

RESULTS

Disease description and pathogen isolation. Wilting symptoms on castor bean were observed in 1991 in Costa Rica and in 1992 in Brazil, in locations where susceptible (N382) and resistant (C8, N7) lines were sown. The wilting of the susceptible line sometimes occurred very early, 1 month after sowing, and symptoms continued throughout the growing season.

Young plants (about 1 month old) wilted and died very quickly without any other aboveground symptoms. On older plants, leaf color often changed, becoming yellow-brown or bronzed, sometimes with a necrotic zone on the leaf edge. Early maturity was commonly observed on these stunted plants. Plants also wilted without any other external symptom above the soil surface. Symptoms included a brown spot that spread around the stem at, or slightly above, ground level and black lesions on the secondary roots. On necrotic roots, the epidermis peeled off and pycnidia could be observed. Spores and pycnidia were characteristic of *M. phaseolina.* No other fungal fructifications could be observed on stunted plants.

M. phaseolina was consistently isolated from wilted plants in the different diseased tissues: small necrotic lateral roots, diseased tap root, and basal stem brown spot from samples collected at Las Palmas and Liberia, Costa Rica, and Xique-xique, Brazil. *Fusarium solani* also was isolated frequently from diseased root tissue, but isolates did not cause wilt symptoms in inoculation tests (data not shown).

Virulence and pathogenicity tests. Seven isolates of *M. phaseolina* from castor bean were inoculated on young (10-day-old) seedlings of the susceptible cultivar (382I) by means of the stem tape technique (Fig. 1). Virulence of the different isolates appeared highly variable (Table 1). Some isolates (no. 166 and no. 199) appeared highly virulent; others did not induce death of inoculated plants.

Comparison, by means of the stem tape inoculation technique, of host range and pathogenicity of two isolates of *M. phaseolina,* French AJF isolate (isolated from sunflower) and the Brazilian isolate (no. 199), demonstrated that host spectrum was similar. Lesion length was greater on French bean, pea, and sorghum when inoculation was done with the *M. phaseolina* Brazilian isolate than when the sunflower isolate was used (Table 2). Three to 4 weeks after inoculation the Brazilian isolate formed pycnidia on castor bean.

Castor bean resistance evaluation. Resistance to *M. phaseolina* of two castor bean genotypes (N7, C8) having high field resistance to the seedling wilt, and one susceptible genotype (N382), was analyzed by using different inoculation methods. The comparison among genotypes was carried out by using isolate no. 199. Resistance was observed throughout the castor bean growing period from germination to fructification. Depending on the stage of plant development, different inoculation test methods were used: soil (germination stage), blotting paper (6-day-old seedlings), stem tape, or toothpick (30-day-old plantlets to 5-month-old flowering plants) (Fig. 1).

In all experiments, genotypes that were field resistant to wilting appeared significantly more resistant to *M. phaseolina* than the field-susceptible genotype (Table 3). All four different inoculation methods used were suitable for evaluation of castor bean resistance to this fungus.

DISCUSSION

Two wilt diseases have been reported on castor bean (32). *Fusarium* wilt resistance of castor bean has already been characterized (23). However, castor bean resistance against sclerotial wilt caused by *M. phaseolina*, the other important soilborne pathogen of this crop, has never been described in the literature. Control of sclerotial wilt through the use of chemicals and cultural practices has been attempted recently, but control of soilborne fungi by these methods remains difficult (12).

Field resistance to castor bean wilt was characterized in a small-seeded *Persicus* sp. from Madagascar. This resistance was integrated in castor bean lines by crosses under naturally infested field conditions. The resistance was effective in warm and dry locations of South Africa, Costa Rica, and Brazil. Field symptoms, in trials containing susceptible and resistant genotypes in 1991 at two locations in Costa Rica and in 1992 at one location in Brazil, were similar to those of sclerotial wilt caused by *M. phaseolina.* Typical pycnidia of *M. phaseolina* were consistently observed on the root system and the basal stem lesion of the susceptible wilted plants. Isolation experiments confirmed that *M. phaseolina* was the most frequently recovered fungus on wilted plants.

Virulence of *M. phaseolina* isolates was studied by means of the stem tape inoculation technique. Variation in the virulence of isolates was evident. Some highly virulent isolates caused rapid seedling death. The pathogenic variability of this fungus has been already described on different crops (13), and this characteristic was proposed for use in the classification of *M. phaseolina* isolates (5).

Field experiments under naturally infested conditions are usually used to evaluate cultivar resistance (2,4,9). Field infection level can be increased by adding highly susceptible cultivars in the experiment (17,18,22). However, *M. phaseolina* virulence is related to soil temperature. Host water stress is another factor favoring development of the disease (7,16). To avoid inconsistencies in field experiment results, different inoculation techniques sufficiently reliable to reflect field resistance were used to evaluate cultivar resistance to *M. phaseolina* in many crops.

In these studies, castor bean lines resistant and susceptible to seedling wilt in the field were inoculated with highly virulent isolates of *M. phaseolina* by means of dif-

Table 3. Castor bean genotype reaction to *Macrophomina phaseolina*, evaluated by four inoculation methods under controlled environmental conditions

	Inoculation method								
	Soil inoculation[t]		Blotting paper	Stem tape	Toothpick[u] Stage				
	Plant weight	Plant death	Root lesion	Stem lesion	Vegetative	Flowering		Fructification	
Genotypes	(% control)[v]	(%)[v]	length (%)[w,x]	length (cm)[x]	15[y]	15	30	15	30
382I (field susceptible)	22.9 a[z]	91.7	100 a	4.5 a	5.85 a	8.26 a	18.17 a	26.53 a	97.65 a
C8 (field resistant)	72.7 b	33.3	8.37 b	2.07 b	1.75 b	4.16 b	8.80 b	9.07 b	32.59 b
N7 (field resistant)	74.1 b	25	17.65 b	2.01 b	2.70 b	3.22 b	6.11 b	11.19 b	39.30 b
Genotypes plant number	n = 24	n = 24	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10

[t] Soil inoculated with 0.8×10^3 propagules per g of soil.
[u] Resistance is assessed by measurement of necrosis length (cm).
[v] Scoring carried out 21 days after inoculation.
[w] Resistance is assessed by measurement of the percentage of root lesion length.
[x] Scoring carried out 15 days after inoculation on 31-day-old seedling.
[y] Days after inoculation.
[z] Means followed by the same letter are not significantly different at $P = 0.05$, using the Newman-Keul's test.

ferent inoculation techniques. Different methods have been described to evaluate *M. phaseolina* resistance of different crops, including sorghum (4,20,24), sesame (6,9), maize (28), sunflower (2,26), cowpea (29), guayule (21), soybean (9), chickpea (25), and beans (1). Two methods, including tissue wounding, were usually used: the toothpick inoculation method in greenhouse and field experiments (3,4,14,27,28), and the stem tape inoculation method in greenhouse experiments (11,15,20,25,31). These techniques do not remotely simulate the natural infection processes, in contrast to the soil inoculation method, which was used for maize and sesame resistance analysis (6,27). All of these methods are used for *M. phaseolina* resistance screening in different crops to increase the efficiency of breeding programs by reducing field testing expenses and length of time for evaluations.

M. phaseolina resistance of castor bean lines expressing resistance and susceptibility to seedling wilt in the field was evaluated by means of three inoculation techniques. These lines were also evaluated with a very early screening technique described to analyze chickpea resistance to *M. phaseolina* (26). This last method, the blotting paper method, done on very young seedlings, avoids tissue wounding and allows resistance assessment in a very short time.

Experimental results obtained with the four inoculation methods (toothpick, stem tape, infested soil, and blotting paper), clearly demonstrated that castor bean resistance to seedling wilt in the field confers resistance to *M. phaseolina*. Genotypes that were field resistant to seedling wilt appeared consistently more resistant to *M. phaseolina* by the different inoculation methods. Castor bean resistance to *Macrophomina phaseolina* is expressed at different stages of plant development, from germination to fructification. However, artificial plant inoculation showed that the resistance was not a immunity-type resistance.

Even though all these inoculation techniques are effective, germination or seedling inoculation methods saved time and appeared more useful. Indeed, resistance evaluation could be evaluated 17 to 22 days after sowing in the soil inoculation method and blotting paper inoculation methods, respectively, and from 45 days to 6 months for other methods.

The results obtained in this work demonstrate for the first time that a high level of *M. phaseolina* resistance exists within the castor bean genome. This result is important in regard to the worldwide distribution of this pathogen, with *M. phaseolina* occurring wherever the castor bean is grown. This fungus is considered to be the most serious castor bean pathogen in dry and warm locations.

Our findings suggest that, instead of chemicals, genetic resistance could be used to limit *M. phaseolina* disease severity on castor bean. The different methods described in this research can be easily used for screening and analysis of castor bean resistance to sclerotial wilt. Attempts are underway to analyze the genetic components of castor bean resistance to *M. phaseolina* originating from the wild species *Ricinus persicus*.

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