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RESEARCH PAPER

Powdery mildew (*Sphaerotheca fuliginea*) resistance in melon is selectable at the haploid level

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Abstract

The major cause of powdery mildew in melons (*Cucumis melo* L.) is the fungus *Sphaerotheca fuliginea*. There are several cultivar- and season-specific races of this fungus. In order to control powdery mildew, it is important to introduce resistance to fungal infection into new cultivars during melon breeding. Haploid breeding is a powerful tool for the production of pure lines. In this study, it was investigated whether powdery mildew resistance could be manifested at the haploid level from two disease-resistant melon lines, PMR 45 and WMR 29. The effects of various races of *S. fuliginea* on diploid and haploid plants of PMR 45 and WMR 29 and of a disease-susceptible line, Fuyu 3 were measured. The responses of haploid and diploid plants to powdery mildew were identical. In addition, haploids that were generated from hybrids between Fuyu 3 and disease-resistant lines were examined. Seven out of 13 haploids from a Fuyu 3 × PMR 45 cross and 10 out of 12 haploids from a Fuyu 3 × WMR 29 cross were classified as resistant plants because they showed the same responses as their disease-resistant diploid parents to the various fungal races. These results indicate that resistance in PMR 45 and WMR 29 is selectable at the haploid level. All of the plant responses were observed by microscopy. A possible mechanism for generating powdery mildew resistance in two different melon lines is discussed.

Key words: *Cucumis melo*, haploid breeding, powdery mildew resistance, *Sphaerotheca fuliginea*.

Introduction

Powdery mildew is a severe disease that causes substantial losses in melon (*Cucumis melo* L.) production around the world (Sitterly, 1978). *Erysiphe cichoracearum* DC ex Merat and *Sphaerotheca fuliginea* (Schlecht ex Fr.) Poll. cause powdery mildew in melons. Both of these races were identified in France (Epinat *et al.*, 1993) and Sudan (Mohamed *et al.*, 1995), but only *S. fuliginea* has been observed as an agent of powdery mildew in the USA (McCreight *et al.*, 1987), Israel (Cohen and Eyal, 1995) and Japan (Hosoya *et al.*, 1999, 2000). *E. cichoracearum* appears to be less important than *S. fuliginea* (Robinson and Decker-Walters, 1997) as a pathogen of melons.

Seven physiologically distinct races of *S. fuliginea* and two races of *E. cichoracearum* were identified on eight different genotypes of melon in France (Bardin *et al.*, 1999). Using the same melon genotypes, three known (1, 2US and 5), and four unknown (N1, N2, N3, and N4) races of powdery mildew were identified in Japan (Hosoya *et al.*, 1999, 2000). The predominating race of powdery mildew changes depending on the melon cultivar, the cultivation season and the geographical area studied (Hosoya *et al.*, 2000). For example, race N2 predominated on cultivar HN21, which is susceptible to races 5 and N2, during the early cultivation season in May. On the other hand, races 1, 2US, and N1 predominated on cultivar Earth Miyabi Natsu 2, which is susceptible to race 5, during the late cultivation season in August. These observations demonstrate rapid succession, whereby specific fungal races prevail for a short time over a small area. Therefore, it is necessary to monitor the predominating races in the field in order to introduce the appropriate resistances into the identified races via breeding programmes.

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The haploid breeding technique is useful for the rapid production of pure lines. Two techniques for producing haploid plants are currently available: anther androgenesis, in which embryos develop from pollen; and parthenogenesis, in which embryos develop from an unfertilized egg cell (Bhojwani and Razdan, 1983). Both techniques have been applied to produce haploid plants from *Cucumis*. Asexual embryogenesis and plantlet development in anther cultures of *Cucumis sativus* L. have been reported (Lazarte and Sasser, 1982). Parthenogenic haploid production was first demonstrated in melons (Sauton and Dumas de Vaulx, 1987); haploid plants were obtained by culturing unfertilized embryos that were pollinated with pollen that had been irradiated with soft X-rays. Parthenogenic haploid production is a reproducible and reliable technique for melons, although the efficiency of haploid production is low, and another deficiency of this technique is the low rate of doubling. In spite of intensive efforts, the efficiency of haploid production has not been improved for many years (Sauton, 1988; Przyborowski and Niemirowicz-Szczytt, 1994). However, the method for doubling the number of haploid plants has improved recently (Koksal *et al.*, 2002; Yashiro *et al.*, 2002). Although the rate has increased by almost 100%, doubling times remain long. Therefore, it is important to select a plant with the desired traits while it is in the haploid stage.

In this study, it was investigated whether the powdery mildew resistance of two resistant melon lines, PMR 45 and WMR 29 was selectable at a haploid level. In order to achieve this goal, the responses of diploid and haploid plants from PMR 45 and WMR 29 and a susceptible line, Fuyu 3, to several races of *S. fuliginea* were observed. In addition, fungal pathogenesis for melon cells was observed by light microscopy. The results allow speculation on potential resistance mechanisms.

Materials and methods

S. fuliginea races used in the inoculation tests

Four races of *S. fuliginea*, 1, 5, N1, and N2 (Hosoya *et al.*, 1999), were used. Races N1 and N2 behave differently on different genotypes of melon (Bardin *et al.*, 1999), and the response pattern is stable more than 4 years after isolation. Therefore, N1 was denoted as race 6, and N2 as race 7.

Plant materials

Three genotypes of melon (*Cucumis melo* L.), Fuyu 3, PMR 45 and WMR 29, were used. Fuyu 3 is susceptible to *S. fuliginea* races 1, 5, 6, and 7. PMR 45 is resistant to races 1 and 6, but not to races 5 and 7. WMR 29 is resistant to races 1, 6, and 7, but not to race 5. F₁ plants were produced from Fuyu 3×PMR 45 and Fuyu 3×WMR 29 crosses. The plants were grown in growth chambers at 26 °C and 16/8 h light/dark.

Haploid plant production

Haploid plants of each genotype were produced by the modified method of Sauton and Dumas de Vaulx (1987) in August 1998 and 2000. Male flowers of a momordica-type melon (*C. melo* L. var.

momordica) grown in a greenhouse were harvested on the day of flowering and irradiated with soft X-rays (130 kR or 65 kR in 1998, and 65 kR in 2000) using a soft X-ray unit (OM-100RA, Ohmicron, Japan). Irradiated pollen was used to pollinate plants of each genotype. Immature ovules were collected from the fruit 3 weeks after pollination and cultured under 16 h light cycles at 25 °C on MS medium (Murashige and Skoog, 1962). Germinated plants were subcultured into MS medium and propagated for use in the following experiments. The relative nuclear content of regenerated plants was analysed by flow cytometry (Ploidy Analyser, Partec, Munster, Germany) according to the manufacturer's protocol, and haploid plants were selected. The seeds of diploid plants were sown *in vitro* on MS medium and propagated as haploid plants. The rooted plants were acclimatized in a growth chamber (12/12 h light/dark cycles at 26 °C).

Inoculation test

Inocula of the *S. fuliginea* races 1, 5, 6, and 7 were maintained on Fuyu 3 cotyledons in a Petri dish under conditions of 16/8 h light/dark at 23 °C. The third expanded leaves were cut from the tips of haploid and diploid plants, sprayed with 70% ethanol for surface sterilization, and wiped with cotton. Leaf discs (1.5 cm in diameter), were excised and placed in a Petri dish containing MS medium. The conidia were blown through the hole at the top of a sedimentation tower using a Pasteur pipette. The Petri dishes were then covered and incubated with 16/8 h light/dark cycles at 23 °C. Susceptibility and resistance phenomena were observed visually within 7–10 d after inoculation.

Observations of the infection process

The third expanded leaf from the apex of each plant was surface-sterilized as described previously, and cut into 4–6 pieces. The pieces were placed in MS medium in a Petri dish. Conidia were removed with a brush from a single colony on a cotyledon and deposited onto a single leaf segment. The Petri dishes were incubated under conditions of 16/8 h light/dark at 23 °C. Leaves were removed at 4, 24, 48, and 120 h after inoculation, and cut into small segments, which were then decolorized in 99% ethanol for 15 min at 95 °C and stained with 0.05% trypan blue in equal volumes of lactic acid, glycerin, phenol, and water for 5 min at 95 °C. Stained segments were examined under a light microscope.

Results

Haploid production

Haploid Fuyu 3, PMR 45 and WMR 29 plants were obtained in 1998, and haploids of the F₁ hybrids were generated in 2000. Greenish embryos emerged at 3 weeks and thereafter, when the ovules were cultured on MS medium. Each embryo was transplanted from a Petri dish to a test tube. The embryos formed young plants with small true leaf-like leaves and roots. Once the plants had developed more than three leaves, the ploidy level was checked by the method described previously. The number of haploids obtained were 6, 10, 12, 51, and 26 on Fuyu 3, PMR 45, WMR29, Fuyu 3×PMR 45, and Fuyu 3×WMR 29, respectively. The haploid production rates ranged from 0.16% to 0.48% of cultured ovules, and were almost identical in 1998 and 2000. Higher rates were seen with PMR 45 (0.31%) and hybrids between Fuyu 3×PMR 45 (0.48%). The haploid production rate in this study was

Table 1. Responses of diploid and haploid plants to four races of *S. fuliginea* and histology of the stages of infection

Genotype	Ploidy	No. of plants tested	Responses and shape of conidia			
			Race 1	Race 5	Race 6	Race 7
Fuyu 3	Diploid	1	S ^a (c) ^c	S (c)	S (c)	S (c)
	Haploid	1	S (c)	S (-)	S (c)	S (c)
PMR 45	Diploid	1	R ^b (g) ^d	S (c)	R (g)	S (c)
	Haploid	2	R (g)	S (-) ^e	R (g)	S (c)
WMR 29	Diploid	1	R (g)	S (c)	R (g)	R (g)
	Haploid	2	R (g)	S (-)	R (g)	R (g)
Fuyu 3×PMR 45	Diploid	5	R (g)	S (-)	R (-)	S (-)
	Haploid	7	R (g)	S (-)	R (g)	S (c)
Fuyu 3×WMR 29		6	S (c)	S (-)	S (c)	S (c)
	Diploid	5	R (g)	S (-)	R (-)	R (-)
	Haploid	10	R (g)	S (-)	R (g)	R (g)
		2	S (c)	S (-)	S (c)	S (c)

^a S: susceptible.

^b R: resistant.

^c c: hyphal development and conidial initiation.

^d g: germ-tube and haustorium formation.

^e -: Not tested.

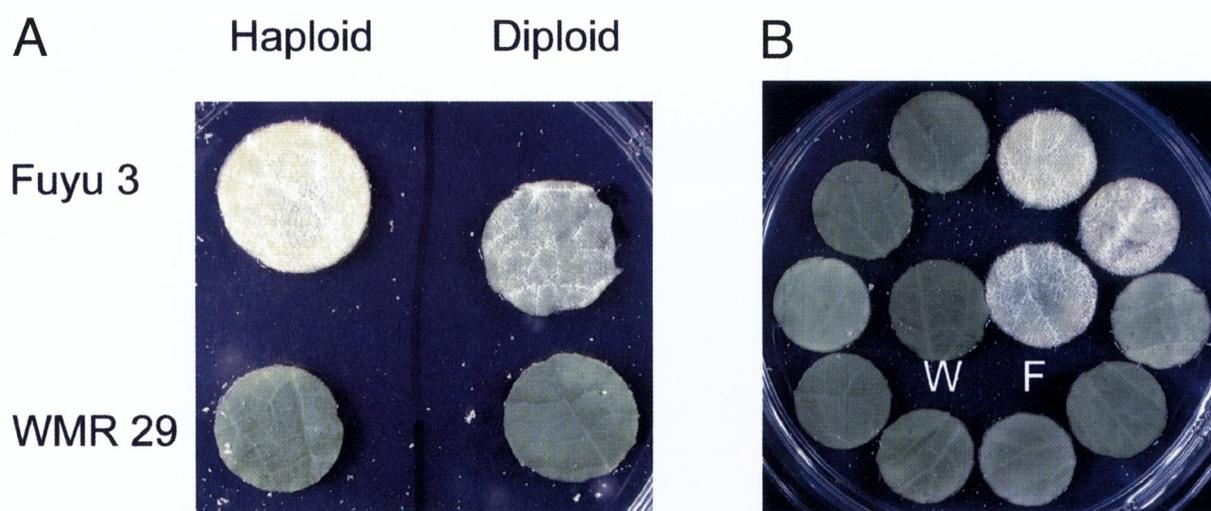


Fig. 1. (A) Responses to *S. fuliginea* race 1 of leaf discs from haploid (left) and diploid (right) plants of Fuyu 3 and WMR 29. (B) Responses to *S. fuliginea* race 1 of leaf discs from diploid plants (middle) of Fuyu 3 (F), and WMR 29 (W), and haploid plants (round) that were generated from the F₁ progeny of a Fuyu 3×WMR 29 cross.

lower than that reported as 0.25% by Sauton and Dumas de Vault (1987), possibly due to differences in the genotypes and physiological states of the mother plants (Sauton, 1988).

Responses of pure melon lines to four races of *S. fuliginea*

The responses of diploid and haploid plants of each genotype to *S. fuliginea* races 1, 5, 6, and 7 are summarized in Table 1. The haploid responses paralleled those of the diploid parents. Both the diploid and haploid forms of Fuyu 3 were susceptible to all three races. PMR 45 diploids and haploids were resistant to races 1 and 6, but were

susceptible to races 5 and 7. Diploid and haploid WMR 29 plants were resistant to races 1, 6 and 7, but were susceptible to race 5. The responses of each genotype were equal in severity, irrespective of their ploidy level (Fig. 1A). In conclusion, the responses of haploid and diploid plants to four *S. fuliginea* races were similar.

Responses of hybrids and hybrid-derived haploids to *S. fuliginea*

The responses to *S. fuliginea* of haploid F₁ plants from crosses between Fuyu 3 and either PMR 45 or WMR 29 are shown in Table 1. The Fuyu 3×PMR 45 F₁ hybrids were similar to the parental PMR 45, in that they were resistant

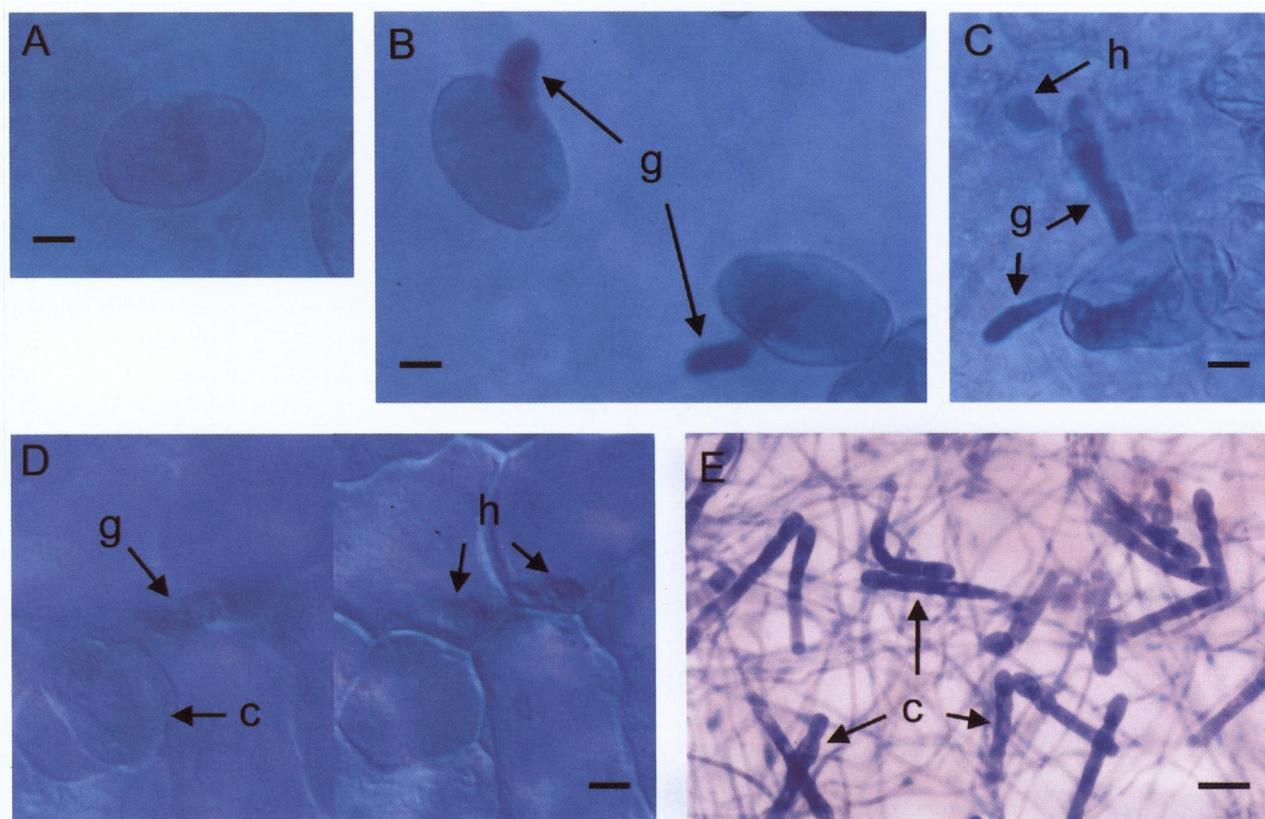


Fig. 2. Histological observations of *S. fuliginea* infection of leaf discs from diploid melon plants. (A) Inoculated conidia from a leaf disc of Fuyu 3. Scale bar=10 μ m. (B) Germ-tube development 4 h after inoculation of a leaf disc of Fuyu 3. Scale bar=10 μ m. (C) Haustoria formation at 24–48 h after inoculation of a leaf disc of Fuyu 3. Scale bar=10 μ m. (D) Multiple haustoria, 48–120 h after inoculation of a leaf disc of PMR 45. Scale bar=10 μ m. (E) Hyphal development and new conidia initiation on a leaf disc of Fuyu 3, 120 h after inoculation. Scale bar=50 μ m. g: Germ tube; h: haustorium; hp: hyphae; c: conidia.

to *S. fuliginea* races 1 and 6, but not to races 5 and 7. The observed resistance to race 1 corroborates previous evidence that the resistance gene is dominant (Epinat *et al.*, 1993). It also suggests that the genes that encode resistance to *S. fuliginea* races 1 and 6 are equally dominant in PMR 45. F₁ hybrids of Fuyu 3 \times WMR 29 were similar to the parental WMR 29 in that they showed resistance to *S. fuliginea* races 1, 6, and 7, but not to race 5. The observed resistance to race 1 supports a previous report that the resistance gene of WMR 29 is dominant (Epinat *et al.*, 1993). In addition, the results suggest that the genes that specify resistance to races 1, 6, and 7 are equally dominant in WMR 29.

The responses of haploid plants that were generated from F₁ hybrids between Fuyu 3 and either PMR 45 or WMR 29 are shown in Table 1 and Fig. 1B. Six of the haploid Fuyu 3 \times PMR 45 hybrids were as susceptible as the susceptible parent Fuyu 3 to fungal races 1 and 6, while seven of these hybrids were as resistant as their resistant parent PMR 45 to races 1 and 6. Two of the haploid plants generated from Fuyu 3 \times WMR 29 crosses were susceptible to races 1, 6, and 7. On the other hand, 10 of the Fuyu

3 \times WMR 29 haploids were as resistant as WMR 29 to races 1, 6 and 7.

Histological observations of S. fuliginea infection of leaf discs

S. fuliginea infection and the resistance of different genotypes of melons were observed (Fig. 2; Table 1).

When Fuyu 3 was inoculated with four different races of *S. fuliginea*, conidial germination occurred after 4 h. Germ tubes and haustoria were observed after 24 h, the mycelia elongated after 48 h, and new conidia appeared after 120 h. There were no differences between diploid and haploid Fuyu 3 plants in terms of the infection process. When PMR 45 was inoculated with four different races of *S. fuliginea*, conidial germination was observed after 4 h. Germ tubes and haustoria, or in some cases multiple haustoria, were observed 48 h after the plants were inoculated with the resistant races 1 and 6. No mycelial elongation was observed even after 120 h. On the other hand, when the PMR 45 plants were inoculated with susceptible races 5 and 7, germ-tubes and elongated mycelia, as well as new conidia, were observed after 120 h. There were no

differences between diploid and haploid PMR 45 plants in terms of the infection process. When WMR 29 was inoculated with races 1, 6, and 7, the development of conidia and the elongation of germ tubes and houstoria were the same as those seen in the resistant response of PMR 45. No mycelial elongation was observed even after 120 h. The responses in germ tube and mycelial elongation, and initiation of new conidia that were seen with Fuyu 3 and PMR 45, were also noted when WMR 29 was inoculated with susceptible race 5. There were no differences in the responses of diploid and haploid WMR 29 plants.

In summary, no differences in infection or resistance mechanisms were noted between diploid and haploid plants of the same genotype (Table 1). The resistant responses of PMR 45 and WMR 29 appeared to emerge after the assembly of haustoria in the epidermal cells. The processes of conidial development in susceptible and resistant haploid plants from Fuyu 3×PMR 45 and Fuyu 3×WMR 29 crosses were the same as those seen in their respective susceptible and resistant parents.

Discussion

Plants develop resistance to disease by introducing structural modifications, such as cell wall thickening or as necrotic defence reaction, or by the production of fungal or bacterial inhibitors (Agrios, 1988). The resistant haploid plants showed significant resistance to powdery mildew although the cell walls of resistant haploids were thinner than those of susceptible diploids (data not shown). Histological observations revealed that conidia germinated and penetrated the epidermal cells of both susceptible and resistant genotypes, leading to the production of haustoria 24–48 h after inoculation. However, this seemed to arrest farther development on resistant diploids and haploids, suggesting that the processes that inhibit hyphal development, such as cell death or the production of inhibitors, might originate in the epidermal cells of the resistant genotype. Powdery mildew of barley is caused by *Erysiphe graminis* and resistance to this disease is encoded by the *Mlo* (Jørgensen, 1992) and *Mla* (Boyd *et al.*, 1995) loci. The *Mlo* genes mediate resistance by directing the formation of subcellular cell wall appositions at the sites of infection, which prevents the penetration of the fungus into the epidermal cells (Jørgensen, 1977, 1992; Wolter *et al.*, 1993). Hypersensitive cell death (HR) in the leaves of plants with the *Mla* locus is an incompatibility response that is thought to mediate resistance by preventing both conidial development at the haustorial stage and hyphal elongation (Koga *et al.*, 1990; Boyd *et al.*, 1995). Therefore, it is likely that PMR 45 and WMR 29 resist powdery mildew by preventing the development of secondary mycelia.

The resistances of PMR 45 (to race 6) and WMR 29 (to races 6 and 7) appeared to be dominant (Table 1). Haploid plants from Fuyu 3×PMR 45 crosses were resistant to races 1 and 6, which suggests that the resistance genes are highly linked in PMR 45. Haploid plants from Fuyu 3×WMR 29 crosses were resistant to race 1, and were also shown to be resistant to races 6 and 7, suggesting that the genes specifying resistance to races 1, 6 and 7 are highly linked in WMR 29. The WMR 29 genes that encode resistance to races 1 and 2 are reported to be linked (Epinat *et al.*, 1993). Studies on the F₂ segregation of responses to *S. fuliginea* may elucidate the relationships between resistance genes.

Although seven haploid plants from the F₁ progeny of Fuyu 3×PMR 45 crosses were resistant to races 1 and 6, six haploid plants were not. The segregation ratio of susceptible:resistant (S:R) plants corresponded to 1:1 ($\chi^2=0.08$, $df=1$, $P>0.05$). This segregation ratio corroborates a previous report in which resistance to race 1 was specified by a single dominant locus (Epinat *et al.*, 1993). Ten out of 12 haploid plants from the F₁ progeny of Fuyu 3×WMR 29 crosses were resistant to races 1, 6, and 7. The S:R ratio was not 1:1 ($\chi^2=5.33$, $df=1$, $P<0.05$), although WMR 29 resistance to race 1 has been reported as being controlled by a single dominant gene (Epinat *et al.*, 1993). Presumably, segregation occurs concomitantly with haploid production and acclimatization in haploid plants from Fuyu 3×WMR 29 crosses. The morphological characterization of haploid plants from F₁ progeny may clarify the nature of this unexpected segregation event. Furthermore, the genetics of resistance genes will provide valuable information.

In conclusion, it has been demonstrated that the resistance of PMR 45 and WMR 29 to powdery mildew caused by different races of *S. fuliginea* is selectable at the haploid level. The production of haploid plants by parthenogenesis is a reproducible and reliable method in melon plants. A combination of techniques should allow the breeding of melons with resistance to powdery mildew. Since PMR 45 and WMR 29 have been used frequently as genetic tools for studying powdery mildew resistance in melons (Pryor *et al.*, 1946; Bohn *et al.*, 1965; Markarian and Downes, 1966), haploid selection can be incorporated into melon breeding programmes.

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