J. Mycopathol, Res, **49(2)** : 265-271 (2011)

Elucidation of host-pathogen interaction using muskmelon callus and *Fusarium oxysporum* f. sp. *melonis*

PIOUS THOMAS[1*] AND M. K. NAIK[2]

[1]Division of Biotechnology, and [2]Division of Plant Pathology, Indian Institute of Horticultural Research, Hessarghatta Lake, Bangalore 560 089, Karnataka,

[1]Present address :Department of Plant Pathology, University of Agricultural Sciences, College of Agriculture, Raichur 584 101, Karnataka.

Received : 05. 02. 2010 Accepted : 15. 04. 2011 Published : 24. 10. 2011

Dual culture of muskmelon callus and vascular pathogen *Fusarium oxysporum* f. sp. *melonis* was established using resistant and susceptible cultivars with a view to study if the parenchymatous cortex region plays any role in governing the initial establishment of this pathogen in the two genotypes. Callus of resistant cultivar Arka Jeet displayed similar extent but more intense mycelial growth compared with the susceptible cv. Monoecious-3. Conversely, Monoecious-3 showed significantly more conidia production, delayed or less chlamydospore formation and higher proportion of macroconidia compared with Arka Jeet. Similar results were obtained with the genotype pair Kakri (resistant) and Lucknow Safeda (susceptible). Low conidia production and higher chlamydospore formation on the callus of resistant cultivars were suggestive that the parenchymatous cortex plays a role in governing initial penetration and ingress of the pathogen to the vascular tissue. The fungus could be kept in virulent form on host callus tissue for at least one year suggesting the usefulness of the technique for long-term preservation of this facultative vascular pathogen.

Key words: *Cucumis melo* L, dual culture, disease resistance, *in vitro*, spore production, vascular wilt pathogen

INTRODUCTION

Fusarium oxysporum f. sp. *melonis* is a devastating wilt inciting pathogen of muskmelon (*Cucumis melo* L.) in the major growing areas of the crop (Mas *et al.*, 1981; Palodhi and Sen, 1991; Thomas *et al.*, 1996a; Zink *et al.*, 1990). Unlike most other necrotic pathogens, *F. oxysporum* targets an organized tissue *viz*. vascular system, causing physical blockage in the xylem coupled with toxin and other metabolite production (Beckman and Roberts, 1995). Evidence in different crops suggests that the host resistance mechanism is centered on the vascular stele, restricting the invader outside the vessels while susceptible ones show extensive vascular colonization (Tessier *et al.*, 1990; Gordon *et al.*, 1990). Whether cortex, the parenchymatous tissue lying between the root surface and the stele, which the pathogen needs to traverse to reach the vascular stele, has any role in governing resistance is not clearly understood. Most of the studies on unraveling the mechanism of resistance involved wounding of root or stem (Gordon *et al.*, 1990; Beckman and Roberts, 1995) thereby excluding the host root cortex region.

Dual culture of callus and pathogen, one of the techniques for studying host- pathogen interaction (Miller *et al.*, 1984; Joshi *et al.*, 1988; Griffith and Hedger, 1994; Nath *et al.*, 2001; Burger *et al.*, 2003) provides an experimental system similar to the parenchymatous cortex under controlled conditions uninfluenced by the environment. The resistance reaction of muskmelon to *F. oxysporum* f. sp. *melonis* is known to be modified by temperature (Thomas *et al.*, 1996b). Further, isolates of *F. oxysporum* are known to lose their virulence when cultured continuously on nutrient medium, thus warranting intermittent inoculation of host plants and re-isolation. Tissue cultures are useful for maintaining pure cultures of obligate pathogens in active

Email: piousts@yahoo.co.in; pioust@iihr.ernet.in

form (Ingram, 1980). The present study has been undertaken with the objectives of examining whether parenchymatous cortex in muskmelon plays any role in conferring resistance to *F. oxysporum* f. sp. *melonis* employing callus cultures and to explore the possibility of using the dual cultures for long-term storage of this necrotic pathogen in virulent form.

MATERIALS AND METHODS

Callus and fungal cultures

The studies were carried out using muskmelon (*Cucumis melo* L.) genotypes that are resistant (R; Arka Jeet, Kakri) or susceptible (S; Monoecious-3 and Lucknow Safeda) to *F. oxysporum* f. sp. *melonis* (Thomas *et al.*, 1994; 1996b). Aseptic seedlings were raised on Murashige and Skoog (1962; MS) basal medium using surface sterilized seeds. The hypocotyl was excised to 1 cm (approx.) segments and were cultured in tubes (150 x 25 mm) on agar (8 g l^{-1}) gelled medium comprising of MS constituents (pH 5.8), 1 mg l^{-1} naphthalene acetic acid, 0.5 mg l^{-1} kinetin and 30 g l^{-1} sucrose. This medium was identified to give uniform friable callus from hypocotyl of different genotypes (Thomas, 1989). The cultures were incubated at 26±2°C under 16 h photoperiod (30-40 µE m^{-2} sec^{-1}) provided by cool-white fluorescent tubes. The callus was maintained with bimonthly subculturing to fresh medium under identical conditions. The fungus culture, supplied by the Vegetable Pathology Laboratory, Indian Agricultural Research Institute, New Delhi, was maintained on potato-dextrose-agar (PDA) medium in test-tube slants with fortnightly subculturing.

Inoculation of callus

The callus in tubes two months after previous subculturing was inoculated at the centre by transferring bits of fungal mycelia from 7-day old PDA slants using an inoculation needle. Based on the observation of variable colonization on different replicate units irrespective of the resistant or susceptible genotype, inoculation with conidial spores was attempted. Spore suspension was prepared by adding 5 ml sterile distilled water to 7-day old slants and washing the same with a fine sterile brush. Mycelial pieces were strained through double-layered cheese-cloth and the spore density was determined using a haemocytometer. The suspension was diluted in sterile distilled water so as to contain 10, 25, 50 or 100 spores per 50 µl (one drop) and each callus was inoculated at the center with 50 µl samples while the control tubes were provided with 50 µl sterile water.

Inoculation of nutrient deprived callus

The studies were carried out initially using Arka Jeet (R) and Monoecious-3 (S) genotypes. In order to ensure that the resistant genotype used in the study were truly resistant, an artificial screening employing seed inoculation (Thomas *et al.*, 1994) was conducted at 26±2°C. Callus was initiated from 4 week old healthy seedlings of Arka Jeet or pathogen non-challenged seedlings of Monoecious-3. The hypocotyls from the above *in vivo* seedlings were thoroughly washed and surface sterilized for 8 min using 0.05% $HgCl_2$ containing two drops of Tween 20 per 100 ml. After six rinses in sterile distilled water, the hypocotyl was excised to 1 cm (approx.) segments and they were cultured on callusing medium as earlier.

The callus derived from clean cultures was maintained with bimonthly subculturing to 50 ml medium in culture bottles (110 mm height × 60 mm diameter). About 5 g of friable callus of Arka Jeet or Monoecious-3 from the upper part of stock cultures that was not in direct contact with the medium was transferred to sterile culture tubes (150 × 25 mm) 8 weeks after previous subculturing. The surface was made even using a sterile glass rod and the tissue was starved of nutrients for 3 days. The callus was inoculated at the centre with 25 spores in 50 µl water as above. There were 20 replicate tubes for each genotype while 10 uninoculated tubes served as control. The dual cultures were incubated at 26±2°C under continuous darkness and observed for the following:

i. extent of necrotic reaction or discoloration in comparison to uninoculated control
ii. time taken for the first visible mycelia growth
iii. (a) area covered by the mycelia and (b) the intensity of mycelia growth on callus, at 3 day interval for 15 days employing the following scale:

Score	*(a) Area-wise description*	*(b) Intensity-wise description*
1	Mycelial growth just visible	Mycelial growth just visible

2	< 25% surface area covered	low intensity
3	25-50% surface area covered	medium intensity
4	50-100% surface area covered	high intensity
5	growth extending downward	profuse growth

The area-wise scoring represented the horizontal spread of mycelia on the surface of callus while the intensity-wise description represented the vertical growth as observed through the tube wall.

iv. In the next experiment, extent of sporulation (asexual spores including both micro- and macroconidia) and the production of chlamydospores were assessed employing 20 replicate tubes per genotype. This was done by drawing 2 g inoculated callus from the top and dispersing it thoroughly in 4 ml sterile distilled water (2 drops of Tween-20 per 100 ml) using a glass rod, and determining spore density employing a haemocytometer. Observations were made at 3 days interval up to 15 days using four replicates tubes per day and five observations per sample, thus giving 20 replicate counts per genotype per day. The experiment was repeated once.

The study using nutrient-deprived callus was extended to the genotype pair Kakri (R) and Lucknow Safeda (S).

Use of callus culture for long-term storage of pathogen

The dual culture of Arka Jeet was kept under ambient conditions to test their usefulness for long-term preservation and maintenance of fungus in virulent form without subculturing. The revival was tested at 3, 6 and 12 months from original inoculation of callus by streaking on PDA slants and the pathogenicity was tested on Monoecious-3 by seed inoculation (Thomas *et al.*, 1994). Cultures maintained on PDA slants by subculturing at fortnightly intervals served as the check. Virulence was assessed based on the time taken for first symptom expression and the per cent mortality 4 weeks after inoculation.

Statistical analysis

The experiments were laid out in completely randomized design with two treatments and 20 replicate tubes per genotype. The data comparing R and S genotypes were analyzed employing t- test for each sampling day. Area and intensity scores were subjected to square root transformation while the spore counts were log-transformed before statistical analysis.

RESULTS AND DISCUSSION

Colonization of callus in presence of nutrient medium

In the initial trial employing resistant and susceptible genotypes, erratic mycelial growth on callus was encountered within one genotype and it was not possible to distinguish between R and S genotypes. Both the texture of callus and the presence of medium below influenced the mycelia growth. The firmer the callus, the slower was the rate of mycelial growth while friable or thinner the callus, faster was the colonization. Cross section of inoculated callus revealed that the rate and extent of colonization on the surface was determined by the time taken by the hyphae to reach the nutrient medium traversing the cell layers. While inoculating with sterile loop, there was a possibility of variable inoculum per callus. The use of spore suspension could overcome such problem. Application of 10 or 25 spores per callus appeared better than 50 or 100 spores allowing slower colonization.

Fungal growth on nutrient deprived callus

In the screening comparing Arka Jeet and Monoecious-3 using nutrient starved callus, no apparent necrotic response was seen on the inoculated sets compared with non-inoculated control as was reported in incompatible combinations of *Phytophthora* spp. and callus tissue (Miller *et al.*, 1984) and there was no visible difference between the R and S genotypes. Mycelia appeared in 2 days on Arka Jeet callus and in 3-4 days in Monoecious-3. Both the genotypes showed more mycelial growth with the passage of time. Throughout the 15 day period, area-wise score appeared comparable for both the genotypes ($P > 0.05$ for all days; Figure 1a) while Arka Jeet showed more intense growth compared with Monoecious-3 ($P < 0.05$ days 6, 9, 12 and 15). Similar results were obtained with the genotype pair Kakri and Lucknow Safeda (Figure 1b). The mycelial growth declined after 2 weeks due to dehydration and exhaustion of callus. The results appeared consistent in the repeat experiment.

The count of conidia was significantly low in the

Table 1. Extent of conidia and chlamydospore production in the callus of resistant (R) and susceptible (S) genotypes of muskmelon on different days after inoculation with *Fusarium oxysporum* f. sp. *melonis*.

Days after inoculation	Conidia ($\times 10^3$ ml^{-1})						Chlamydospores ($\times 10^3$ ml^{-1})					
	Arka Jeet (R)	Monoecious-3 (S)	Significance	Kakri (R)	Lucknow Safeda (S)	Significance	Arka Jeet (R)	Monoecious-3 (S)	Significance	Kakri (R)	Lucknow Safeda (S)	Significance
3	237.5	925.0	**	187.5	550.0	**	112.5	0	**	137.5	50.0	*
6	625.0	2362.5	**	1625.0	3475.0	**	175.0	87.5	**	212.5	100.0	*
9	3875.0	4825.0	**	2800.0	4250.0	**	200.0	112.5	*	262.5	137.5	*
12	3075.0	6412.5	**	4375.0	5050.0	NS	250.0	200.0	NS	525.0	275.0	*
15	3087.5	5387.5	**	4625.0	6000.0	**	312.5	225.0	NS	675.0	312.5	**

*, **; Significant at P = 0.01 and 0.05 respectively; NS, Not significant

tissue suspension prepared from Arka Jeet callus compared with Monoecious-3 throughout the 15 days period ($P < 0.05$ for all days; Table 1) and similar results were obtained with the Kakri - Lucknow Safeda pair. Other studies employing dual culture of necrotic fungus and host callus reported slower or less extensive colonization of the resistant plant than susceptible host (Helgeson *et al.* 1976; Miller *et al.*, 1984). The callus from resistant genotypes in the present study showed similar or relatively more mycelial growth than the susceptible types while the sporulation was significantly less. In whole plant systems, sporulation is known to occur in resistant cultivars but this is very low compared to susceptible cultivars while mycelial growth may be similar (Bell and Mace, 1981).

Chlamydospores could be observed from day 3 in Arka Jeet while they were not seen until day 6 in Monoecious-3. Arka Jeet showed more chlamydospores with time with the difference between the genotypes significant on days 6 and 9. Consistent results were obtained in the repeat trial except that the appearance of chlamydospores was delayed to day 9 in Arka Jeet and day 15 in Monoecious-3. Kakri showed significantly higher chlamydospore production than Lucknow Safeda throughout the period of study (Table 1). The earlier and higher production of chlamydospores in Arka Jeet and Kakri compared to susceptible Monoecious-3 or Lucknow Safeda was another indication of an unfavourable environment encountered by the pathogen in the resistant genotypes, inducing it to produce the resting spore which can survive adverse environments (Nelson, 1981).

The observations in this study were suggestive that the parenchymatous cortex plays some role in governing the movement of *F. oxysporum* from root epidermis to the vascular tissue. Accelerated symptom development in cut stem or cut root inoculations compared to root-dip inoculated tomato and peas (Bishop and Cooper, 1983) also suggested that cortex has some role in resisting initial pathogen penetration and ingress to vascular tissue. The results also indicated the scope of using dual cultures for basic studies such as the biochemical changes associated with infection unaffected by environmental conditions. Resistance reaction of muskmelon genotypes to *F. oxysporum* is significantly influenced by environmental factors such as low temperature (Thomas *et al.*, 1994; 1995) and light intensity (Burger *et al.*, 2003).

It was essential that uniform type of tissue, whether firm or friable, be used in both resistant and susceptible genotypes. The hormonal regime, explant source and genotype could alter the texture of the callus which in turn could modify the response to the pathogen (Thomas, 1989). The more intense mycelial growth in Arka Jeet could be due to softer texture of callus compared with Monoecious-3. It

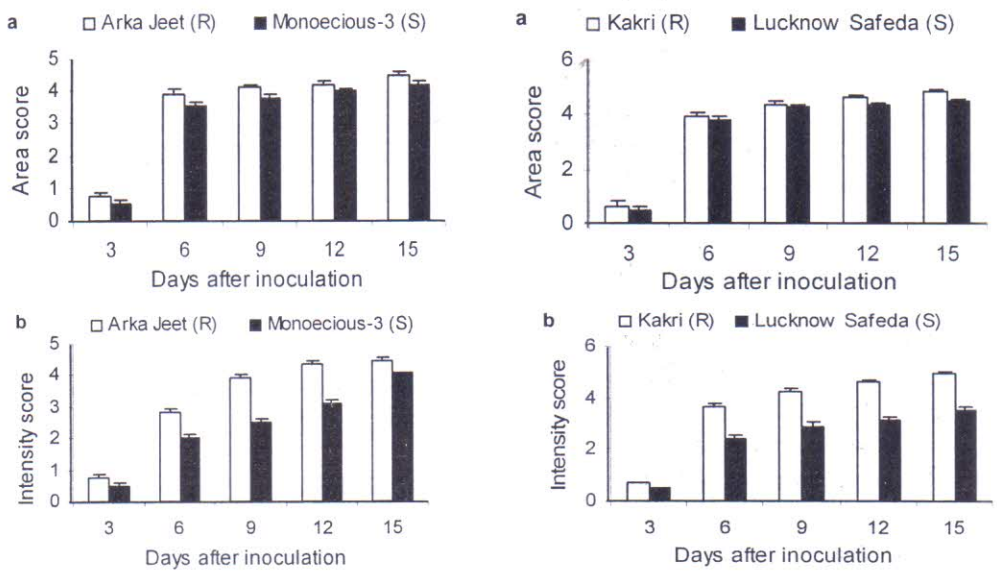

Fig. 1. Extent of colonization by fungal mycelia on muskmelon callus based on the area (a) or intensity of mycelia growth (b) in the resistant (R)-susceptible (S) genotype combinations Arka Jeet and Monoecious-3 (*left*), and Kakri and Lucknow Safeda (*right*) recorded on different days after inoculation with *Fusarium oxysporum* f. sp. *melonis*. (Bars indicate standard error).

was essential that the callus be removed from the nutrient medium and relatively low inoculum (25 spores / callus) be employed to obtain the differential response.

An opposite trend between mycelial growth and sporulation was noticed in this study in that the cultures with more intense mycelia growth often showed less conidia production. Similar results were obtained with the *F. oxysporum* f. sp. *melonis* culture grown on PDA supplemented with yeast extract (10 g l^{-1}) leading to faint mycelia growth and a four folds increase in spore production over regular PDA (Thomas, 1989). The resistant genotypes showed delayed appearance and lower proportion of macroconidia compared with the respective susceptible check (Figure 2a, b). The significance of this observation remains to be elucidated.

Use of callus for long-term storage of pathogen

The inoculated callus, irrespective of genotype, was reduced to a small dry mass within 3 months owing to nutrient consumption by the fungus and tissue dehydration. The fungus was retrieved from the dry callus after 3, 6 and 12 months. These cultures showed virulence comparable to or better than the original cultures. Using culture retrieved from callus after 12 months, first symptom of seedling drooping in Monoecious-3 was observed by fourth day after emergence followed by complete seedling mortality by 2 weeks. The PDA maintained cultures showed slightly delayed symptoms but per cent mortality 4 weeks after planting was comparable to the former.

Callus cultures have been employed for the storage of pure cultures of obligate pathogens (Ingram, 1980) where the tissue should remain healthy to support their growth. *F. oxysporum* can survive on crop debris for long periods. Callus cultures in the present study maintained the pathogen in virulent form for long periods without the need for sub-culturing. This approach helps in long-term storage of various accessions of fungi with minimum chance for cross contamination losses, variation in culture, loss of virulence on artificial medium besides saving manpower and other resources.

In conclusion, the callus of *F. oxysporum* f. sp. *melonis* resistant genotypes displayed low conidia production and early chlamydospore production suggesting a possible role of cortex in determining the early events associated with infection and subsequent hyphal movement to the vascular stele. The dual culture system is useful for long-term preservation of pure fungal cultures in virulent form and offers scope for studying the mechanism of host resistance under controlled conditions.

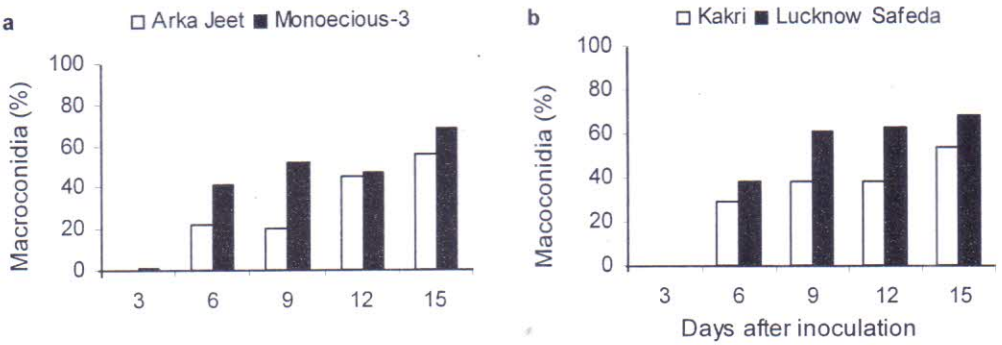

Fig. 2. *T*he proportion of macroconidia to total conidia (micro + macro-conidia) production in the callus of resistant-susceptible muskmelon genotype combinations Arka Jeet and Monoecious-3 (a) and Kakri and Lucknow Safeda (b) recorded on different days after inoculation with *Fusarium oxysporum* f. sp. *melonis*.

ACKNOWLEDGEMENT

The supply of fungal culture by Dr. B. Sen, Indian Agricultural Research institute, New Delhi is gratefully acknowledged.

REFERENCES

Beckman, C. H. and Roberts, E. M. 1995. On the nature and genetic basis of resistance and tolerance to fungal wilt diseases of plants. In: *Advances in Botanical Research vol. 21.* (Ed. by J. H. Andrews and I. C. Tommerup), Academic Press, London, p. 35-77.

Bell, A. A. and Mace, M. E. 1981. Biochemistry and physiology of resistance. In: *Fungal Wilt Disease of Plants*. (Ed. by M. E. Mace, A. A. Bell, and C. H. Beckman), Academic Press, New York, p. 431-436.

Bishop, C. D. and Cooper, R. M. 1983. An ultrastructural study of root invasion in three vascular wilt diseases. *Physiol. Plant Pathol.* **22**: 15-27.

Burger, Y., Katzir, N., Tzuri, G., Portnoy, V., Saar, U., Shriber, S., Perl-Treves, R. and Cohen, R. 2003. Variation in the response of melon genotypes to *Fusarium oxysporum* f. sp. *melonis* race 1 determined by inoculation tests and molecular markers. *Plant Pathol.* **52**: 204-211.

Gordon, T. R., Jacobson, D. J., May, D. M., Tyler, K. B. and Zink, F. W. 1990. Fruit yield, disease incidence, and root colonization of hybrid muskmelons resistant to *Fusarium wilt*. *Plant Dis.* **74:** 778-781.

Griffith, G. W., and Hedger, J. N. 1994. Dual culture of *Crinipellis perniciosa* and potato callus. *Europ. J. Plant Pathol.* **100**: 371-379

Helgeson, J. P., Haberlach, G. T., and Upper, C. D. 1976. A dominant gene conferring disease resistance to tobacco plants is expressed in tissue cultures. *Phytopathol.* **66**: 91-96.

Ingram, D. S. 1980. Tissue culture methods in plant pathology. In: *Tissue Culture Methods for Plant Pathologists* (Ed. by D. S. Ingram and J. P. Helgeson). Blackwell Scientific Publications, Oxford, p. 3-9.

Joshi, M. K., Singh, U. S. and Garg, G. K. 1988. Use of Brassica callus culture to induce sporulation in *Alternaria brassicae* (Berk.) Sacc. *Plant Cell Tiss Org. Cult.* **14**: 59-62.

Mas, P., Molot, P. M., and Risser, G. 1981. Fusarium wilt of muskmelon. In: *Fusarium: Diseases, Biology and Taxonomy*. (Ed. by P. E. Nelson, P. E. Toussoun and R. J. Cook), The Pennsylvania State University Press, University Park, p. 169-177.

Miller, S. A., Davidse, L. C. and Maxwell, D. P. 1984. Expression of genetic susceptibility, host resistance and nonhost resistance in alfalfa callus tissue inoculated with *Phytophthora megasperma*. *Phytopathol.* **74**: 345-348.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-497.

Nath, M. D., Suman, S. and Uma, K. 2001. Growth of *Albugo candida* infected mustard callus in culture. *Mycopathol.* **152**: 147-153.

Nelson, P. E. 1981. Life cycle and epidemology of *Fusarium oxysporum*.. In: *Fungal Wilt Disease of Plants*. (Ed. by M. E. Mace, A. A. Bell and C. H. Beckman), Academic Press, New York. p. 51-80.

Palodhi, P. R. and Sen, B. 1991. Fusarium wilt endangers river bed cultivation of cucurbits. *Indian J. Mycol. Res.* **19**: 51-56.

Tessier, B. J., Meuller, W. C. and Morgham, A. T. 1990. Histopathology and ultrastructure of vascular responses in peas resistant or susceptible to *Fusarium oxysporum* f. sp. *pisi*. *Phytopathol.* **80**: 756-764.

Thomas, P. 1989. *Studies on Fusarium Wilt Resistance in Muskmelon* (*Cucumis melo* L.), Ph. D. thesis, Indian Agricultural Research Institute. New Delhi, India.

Thomas, P., More, T. A. and Sen, B. 1994. Response of subterranean hypocotyl region of seedlings in screening muskmelon (*Cucumis melo* L.) for resistance to *Fusarium oxysporum* f. sp. *melonis*. *Indian J. Agric. Sci.* **64**:194-198.

Thomas, P., More, T. A. and Sen, B. 1995. Influence of soil temperature on wilt of muskmelon incited by *Fusarium oxysporum* f. sp. *melonis* and *Fusarium solani*. *Acta Phytopathol. Entomol. Hungarica* **30**: 175-180.

Thomas, P., More, T. A. and Maini, S. B. 1996a. Screening of muskmelon germplasm for resistance to *Fusarium* wilt and studies on phenols in relation to resistance. *Indian J. Plant Prot.* **24**: 10-14.

Thomas, P., More, T. A. and Sen, B. 1996b. Selection and *in vitro* propagation of muskmelon plants with superior resistance to *Fusarium oxysporum* f. sp. *melonis*. *Trop. Agri.* **73**: 187-191.

Zink, F. W., Thomas, C. E. and Charleston, S. C. 1990. Genetics of resistance to *Fusarium oxysporum* f. sp. *melonis* races 0, 1 and 2 in muskmelon line MR-1. *Phytopathol.* **80**: 1230-1232.