

14.

Improving tolerance to *Fusarium oxysporum* f. sp. *melonis* in melon using tissue culture and mutation techniques

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Abstract

Fusarium wilt is a vascular disease of the Cucurbitaceae family, especially in muskmelon (*Cucumis melo* L.), caused by the soil fungus *Fusarium oxysporum* f. sp. *melonis* (FOM). This pathogen persists in the soil for extended periods of time, and the only effective control is the use of resistant varieties. During the last three decades, tissue culture techniques have been utilized in crop improvement to generate changes in the genetic material of plants via *in vitro* somaclonal variations (by organogenesis or somatic embryogenesis) and induced mutagenesis. More recently, researchers have been using *in vitro* techniques to investigate the effects of fungal culture filtrates or toxins on susceptible and resistant genotypes of different plant species or cultivars to assess disease resistance. This method is effectively used for cucumber and melon. There are various *in vitro* culture techniques that may be used for cucumber (Malepsezy, 1988). In this chapter, we show a method for mass-selection of melon mutants resistant to Fusarium wilt. *In vitro* selection of resistant cells, which will come from irradiated and non-irradiated explants, is done using culture filtrates of different FOM races. This research can lead to the development of new melon cultivars that will be resistant to Fusarium wilt.

Introduction

Fusarium wilt is a vascular disease of the Cucurbitaceae family caused by the soil fungus *Fusarium oxysporum* f. sp. *melonis* (FOM), which is very detrimental to muskmelons (*Cucumis melo* L.). Fusarium wilt of melon is prevalent in temperate and tropical regions and causes a worldwide problem. FOM can survive in the soil for extended periods of time as chlamydospores, and is capable of colonizing crop residues and roots of most crops grown in rotation with melon. The only effective control is the use of resistant varieties. Four races of FOM have been identified, namely 0, 1, 2 and 1.2 (Risser *et al.*, 1976; Mas *et al.*, 1981). Race 1.2 was further subdivided into race 1.2y and 1.2w, which cause yellowing and wilt symptoms, respectively. Two resistance genes (*Fom-1* and *Fom-2*) have been identified in melons (Mas *et al.*, 1981; Martyn and Gordon, 1996; Joobeur *et al.*, 2004). *Fom-1* confers resistance to FOM races 0 and 2, and *Fom-2* confers resistance to races 0 and 1. These two genes are extensively used in breeding programmes, which can be assisted by marker assisted selection using markers linked to these resistance genes (Zheng and Wolf, 2000; Wang *et al.*, 2000; Burger *et al.*, 2003). No genes have been identified that confer resistance to race 1.2 (Zink and Thomas, 1990; Wechter *et al.*, 1995). However, polygenic recessive

genes have been found to confer resistance to race 1.2 in Piboule genotypes (Messiaen *et al.*, 1962; Risser *et al.*, 1976). Table 14.1 shows some sources of resistance to FOM among melon genotypes.

Melon production in Turkey is 1,700,000 tons (Anonymous, 2005) and it is declining the year after year because of Fusarium wilt. Therefore, Fusarium wilt has a high economic importance in the cultivation of muskmelon in Turkey. In some parts of Turkey the prevalent races of this pathogen were determined. Fantino and Zengin (1974) isolated race 1.2 from wilted plants showing intensive root rot in Eastern Thrace. In the Aegean region, Yildiz (1977) recovered three races of the pathogen, race 1 being the most common (57%), followed by race 1.2 (35%) and race 0 (6%). Yücel *et al.* (1994) obtained races 0, and 1-2 in the East Mediterranean region. Erzurum *et al.* (1999) isolated the races 0, 1.2 and 2 in Central Anatolia. Based on these results, Fusarium wilt is a wide spread disease over all regions of Turkey. FOM has caused severe losses for farmers as our native cultivars are not resistant to this disease. It is believed our native cultivars will disappear if resistance to FOM is not introduced into the cultivated material. For this reason, many scientists in Turkey are focusing on research to develop new resistant cultivars via conventional and biotechnological breeding methods.

In vitro techniques became widely spread during the 20th century, and their potential to make important contributions to plant breeding was quickly understood. *In vitro* techniques for crop improvement first consisted of micropropagation and plant regeneration, and then *in vitro* methods were also found to be useful for eliminating disease and selecting for resistant cells or explants.

Through the last three decades, *in vitro* tissue culture techniques have been used to generate genetic changes via somaclonal variations (by organogenesis or somatic embryogenesis) that can be used for breeding purposes. *In vitro* selection using specific chemical compounds and pathogens is another useful aspect of tissue culture (Figure 14.1). Selection with phytotoxins and culture filtrates appears to be more effective than the use of the pathogen itself (Van Harten, 1998). Researchers now use fungal culture filtrates or toxins to investigate the response of susceptible and resistant genotypes of different plant species or cultivars to disease factors. The use of *in vitro* methods for the evaluation of resistance is dependent upon a positive correlation between *in vitro* culture filtrate resistance and whole plant disease resistance. Chawla *et al.* (1987), Gray *et al.* (1986), Connell *et al.* (1990), Malepsezy and El-Kazzaz (1990), El-Kazzaz and Malepsezy (1992) developed protocols for *in vitro* determination of resistance. In comparison with field screening other biotechnological methods, selection techniques are more cost and labour effective and do not require large experimental fields.

More recently, *in vitro* techniques were combined to mutation induction for generating genetic variation, including novel disease resistant mutants. Mutation induction can be caused by chemical or physical mutagens that alter the structure of the DNA (Smith, 1985). Treatment of *in vitro* tissues with physical or chemical mutagens may increase the frequency of genetic variation considerably. The physical mutagens most commonly used are X-rays, gamma rays and UV light, whereas Ethyl Methane-Sulphonate (EMS) is the chemical mutagen most used in crop improvement. Irradiation treatments may be a suitable choice of mutagen for a number of reasons because the application is fast and, in contrast to chemical mutagens, there is no risk that residues remain in the medium. The *in vitro* mutation frequencies are much higher than somaclonal variation. More over somaclonal variation is another important point for *in vitro* plant breeding. You can catch desirable cells or plantlets, which are resistant for disease by this method. Finally, *in vitro* techniques are useful as well in classical mutation breeding programs by vegetative propagation before or after mutagenic treatment, by *in vitro* selection or by clonal propagation of selected mutants (Figure 14.1).

According to Malepsezy and El-Kazzaz (1990), *in vitro* selection using FOM filtrates can be effectively used for the selection of cucumber and melon (Megnegneau and Branchard, 1991). One of the purposes is to use this method for the selection of various mutants, which are resistant to disease. In this research we are going to determine the resistant cells, which will come from irradiated and non-irradiated explants by using races of *F. oxysporum* f. sp. *melonis* culture filtrates *in vitro* conditions. If we will get successful results by this

method we will be able to develop new melon cultivars, which will be resistant to *F. oxysporum* f. sp. *melonis*.

Methodology

The present work was performed with *in vitro* plantlets obtained from seeds of melon cv. Yuva which is an important commercial cultivar in Turkey. Two types of explants (cotyledon and hypocotyls with leaf and cotyledon explants) were used for callus and suspension culture initiation (Taner *et al.*, 2004).

Preparation of the fungal culture filtrate and selection medium

In this research we followed the procedure developed by Megnegneau and Branchard (1991). Petri dishes containing potato dextrose agar (PDA) medium were inoculated with FOM isolates which were named (A-1)₄ and (A-6)₄, which obtained from Ankara University Plant Protection Department in Turkey (Erzurum *et al.* 1999). Petri dishes were incubated at 26°C in the dark. Fifteen days later, 2×10^6 conidia were transferred to 200 ml of liquid Richard medium. Cultures were kept at 26°C in the dark. After twenty days, fungal cultures were filtered twice through filter paper to remove mycelia. The pH of the filtrate was adjusted to pH 5.7 with 1 N HCl or 0.1 N NaOH. Subsequently, cultures were sterilized through a 0.22 µm filter unit attached to a syringe. For filtrate preparation it is very important that to avoid thermal degradation of toxic compounds in the fungal culture filtrate. Therefore, filtrates should be aliquotted and frozen for long-term storage. The working filtrate aliquot can be kept in the refrigerator. Filtrates were added to autoclaved modified MS basal medium. According to Taner (2002) and Taner *et al.* (2004) non irradiated and irradiated cotyledon and hypocotyls with leaf and cotyledon explants were transferred into petri dishes containing half strength of MS medium with basal salts (Murashige and Skoog, 1962) supplemented with 0.5 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetin and 250mg/l casein enzymatic hydrolysate (Sigma) (for cotyledon explants) and MS basal medium, which was supplemented with 0.5 mg/l Indole-3-acetic acid (IAA), 0.5 mg/l 6-Benzylaminopurine (BAP) (hypocotyls with leaf and cotyledon explants), both mediums contain different ratio of filtrate and 15% sucrose. For both media, the pH was adjusted to 5.6 the steps for filtrate preparation are shown in Figure 14.2. The different proportions used in this study were 4, 6, 8, 10, 12, 14, 16, 18 and 20 % (v/v). Control plates contained only the modified MS basal medium.

Preparation of *in vitro* plant material

Figure 14.3 shows the steps for the initiation of the callus cultures. Seeds of melon genotypes were surface sterilized, rinsed and germinated *in vitro* as previously described by Çürük (1999) and Taner (2002). According to the authors seeds were surface sterilized for 20 minutes in 20% sodium hypochloride solution that was involved tween 20 (1ml/100ml) and washed three times in sterile distilled water. Seed cover is removed after sterilization. They are planted in magenta b-cap that is contained 0.50ml solid MS medium containing 0.7% Difco agar and 15% sucrose. The cultures maintained at 25°C, under fluorescent illuminance with a light intensity of 10 000 lux, with 16 h photoperiod. For resistance screening, two kinds of non-irradiated and irradiated explants (i.e., cotyledons and hypocotyls containing leaves and cotyledons) were used according to Taner *et al.* (2004). Cotyledon explants were transferred into Petri dishes containing half strength of MS medium with basal salts (Murashige and Skoog, 1962) supplemented with 0.5 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetin and 15% sucrose. Hypocotyls with leaf and cotyledon explants were transferred MS basal medium, which was supplemented with 0.5 mg/l Indole-3-acetic acid (IAA), 0.5 mg/l 6-Benzylaminopurine (BAP) and 15% sucrose. For both media, the pH was adjusted to 5.6 with 1 N HCl or 0.1 N NaOH. It is very important that the experiments contain more than 50 explants for each combination.

***In vitro* mutagen treatment**

Seven days old *in vitro* plantlets which are containing real leaves and cotyledons irradiated by ^{60}Co gamma source after seed germination. They are irradiated with ^{60}Co gamma rays at five different doses (10, 20, 30, 40 and 50 Gy). Irradiation doses and their effects on the plants are known to be genotype-dependent, so every experiment should start with the determination of the optimal irradiation dose for their genomic material. This is typically calculated as LD_{50} , which corresponds to the 50% survival dose. Doses weaker or stronger than LD_{50} may also be used, but too little mutations may be observed or lethality may be too high, respectively. Even for the same genetic material, the optimal dose for mutation induction *in vitro* is lower than for seed irradiation. In our experiments 25 Gy gamma irradiation dose found an effective dose for *in vitro* plantlets of Yuva cultivar. After irradiation, it is important to transfer the explants in fresh regeneration medium contain filtrate which was described Preparation of *In Vitro* Plant Material section of this chapter to avoid any toxicity of the medium components due to the irradiation.

***In vitro* screening and evaluation**

The fungal culture filtrate was added to the media at different concentrations as indicated above. Cotyledon cultures were incubated at 26°C in the dark for three weeks. At the end of this three weeks period observations made on explants according to their regeneration capacity (Figure 14.4). White, yellow callus formation from cotyledon explants determined survival capacity about explants to filtrate. Hypocotyl with leaf and cotyledon explants were incubated under a light period of 16 h at 15 000 lux at 26°C Taner *et al.* (2004). Regenerated plantlets that showed resistance to the culture filtrate were isolated and sub-cultured every two weeks onto the same medium (these mediums have to contain filtrate for observation of explants survival). Shoot and root regeneration of explants, their growth performance and shoot number are important observation points of our research.

The callus cultures obtained from hypocotyl explants with leaves and cotyledons were maintained for three weeks according to Taner and Yanmaz (2003). We transferred calluses to hormone free MS medium that includes filtrate for somatic embryo regeneration. MS medium supplemented with 1.0 mg/l Indole-3-acetic acid (IAA) is proper medium for plantlet formation from shoot cultures and somatic embryos (Taner 2002). After that period, the rate of mortality of the explants was estimated. Plantlets are then transferred to greenhouses and advanced to the M_2 generation for further selection and evaluation.

Applications

Seven doubled-haploid (DH) melon lines, originated from parthenogenesis using irradiated pollen were produced. Two promising DH lines, were selected for resistance/tolerance to Fusarium wilt following inoculations with race 1.2w. These two doubled-haploid lines represent a source of resistance/tolerance commercially exploitable either as rootstocks or as lines for conventional breeding (Ficcadenti *et al.*, 2002).

Table 14.2 shows the results of previous studies using *in vitro* methods for the selection of melon and cucumber resistant to Fusarium wilt. This technique is very effective for the rapid screening of melon and cucumber. We are currently applying *in vitro* mutagenesis combined to *in vitro* selection for the improvement of the commercial melon cultivar called Yuva to expand the genetic variation of muskmelon in Turkey and to select resistant types from this cultivar. We will plan to use this method for other melon cultivars in Turkey.

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Table 14.1. Muskmelon genotypes resistant to *Fusarium oxysporum* f. sp. *melonis**.

Genotype	Resistance	Type and origin
Védrantais	Resistant to FOM races 0 and 2	Cultivar (INRA, France)
Dulce	Resistant to FOM races 0 and 2	Cultivar (USA)
FM 025	Resistant to FOM races 0 and 2	Breeding line (ARO, Israel)
FM 018	Resistant to FOM races 0 and 2	Breeding line (ARO, Israel)
FM 004	Resistant to FOM races 0 and 2	Breeding line (ARO, Israel)
FM 023	Resistant to FOM races 0 and 2	Breeding line (ARO, Israel)
FM 014	Resistant to FOM races 0 and 2	Breeding line (ARO, Israel)
FM 024	Resistant to FOM races 0 and 2	Breeding line (ARO, Israel)
Doublon	Resistant to FOM races 0 and 2	Cultivar (INRA, France)
Hemed	Resistant to FOM races 0 and 2	Commercial cultivar (Hazera, Israel)
Freeman cucumber	Resistant to FOM races 0 and 1	Breeding line (ARO, Israel)
PI 161375	Resistant to FOM races 0 and 1	Breeding line (ARO, Israel)
F65	Resistant to FOM races 0 and 1	Breeding line (ARO, Israel)
I4-6-2-B	Resistant to FOM races 0 and 1	Breeding line (ARO, Israel)
Maqdimon F1 Hybrid	Resistant to FOM races 0 and 1	Commercial cultivar (Hazera, Israel)
Omega (5080) F1 Hybrid	Resistant to FOM races 0, 1 and 2	Commercial cultivar (Nunza, the Netherlands)
Caruso (5093) F1 Hybrid	Resistant to FOM races 0, 1 and 2	Commercial cultivar (Nunza, the Netherlands)
Piboule	Resistant to FOM race 1-2	Cultivar (France)
Dinero	Resistant to FOM race 1-2	Commercial cultivar (Syngenta Seeds, Milano, Italy)
ASR04993033	Resistant to FOM race 1-2	Commercial cultivar (Asgrow Seeds, Latina, Italy)

*Modified from Burger et al. (2003)

Table 14.2. *In vitro* screening for resistance to Fusarium wilt in *Cucumis* spp.

Specie and Cultivar	Pathogen	Result	Reference
Melon (<i>Cucumis melo</i> cv. Yuva)	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i>	On going study	
Cucumber (<i>Cucumis sativus</i> cv. Borszczagowski and Gy-3)	<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	7% of regenerated plants were resistant	Malepszy and Kazzaz (1990)
Melon (<i>Cucumis melo</i>)	<i>F. oxysporum</i> f. sp. <i>melonis</i>	Correlation between filtrate density and explants growth established	Megnégneau and Branchard (1991)
Cucumber (<i>Cucumis sativus</i> cv. Borszczagowski and Gy-3)	<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	Selected plants showed various modes of resistance expression when exposed to 100% of culture filtrate	Kazzaz and Malepszy (1992)

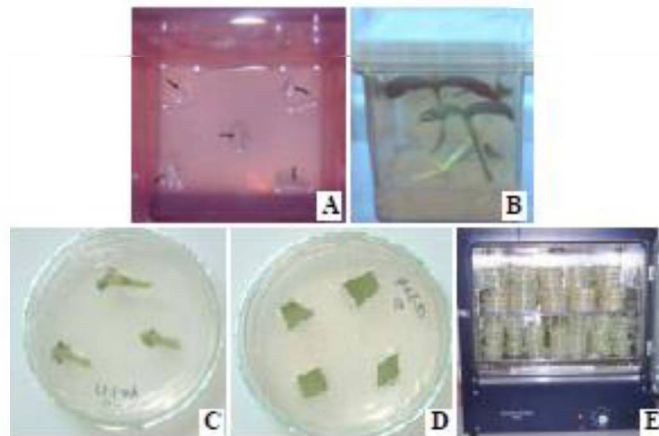


Figure 14.3. The steps for culture initiation. (A) *In vitro* seed culture. Seeds were cultured after removal of the seed cover. (B) *In vitro* plantlet production. (C) Hypocotyls with leaf and cotyledon explants. (D) Cotyledon explants. (E) Incubation period.

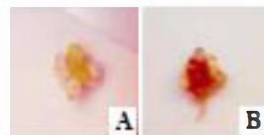


Figure 14.4. Selection for resistant melon calli with fungal culture filtrate. (A) Live callus (resistant); (B) Dead callus (susceptible).