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Full Length Research Paper

Detection of species diversity of arbuscular mycorrhizal fungi (AMF) classic and molecular methods from the melon (*Cucumis melo* L.) plants in Van and its districts

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Arbuscular-mycorhizal fungi (AMF) from melon plants grown in Van province, were studied by nested-PCR method to establish colonization ratio of related fungi in plants and to detect the fungi at species level. From 10 different locations, a total of 100 soil samples were taken from rhizosphere area of melon plants. It was also determined that 52% of these plants were colonized by arbuscular-mycorhizal fungi and were able to establish symbiotic relationship in variable ranges (4.5 to 37.8%) *Glomus intraradices* and *Glomus mosseae* were two identified AMF.

Key words: Arbuscular-mycorhizal fungi (AMF), melon, *Glomus intraradices, Glomus mosseae,* Van.

INTRODUCTION

Turkey is an important melon producer in the world with 1.74 million tons of production on 115 thousands ha area (FAOSTAT, 2008). Moreover, Turkey is located in the secondary gene center for melon lying from minor Asia to Japan (Pitrat et al., 1999). There are rich sources of melon germplasm in Turkey because beside modern production methods, traditional farming techniques relying on their own seed sources are still alive among some small scale melon producers. Therefore, melon germ-plasm collections by various studies in Turkey have been carried out in order to introduce and utilize unique landraces or genotypes for many desirable horticultural characteristics (Kucuk et al., 2002).

One of the melon germplasm collections has been carried out by our team in the Lake Van Basin of Turkey. Lake Van is the largest body of water in Turkey and is the fourth largest inland lake of the world with 3.713 km^{-2} of area (Degens and Kurtman, 1978). It has an interior basin and shores at Central, Edremit, Ercis, Ercek, Gevas and Muradiye towns of Van province (Demir et al., 2006). In Lake Van Basin, besides many agricultural crops and some commercial melon cultivars, many melon landraces are produced in relatively large amounts. Lake Van Basin is also the origin of cantaloupe melon largely produced in France, Germany, Italy and Spain. Zhukovsky (1951) and Gunay (1993) reported that melon had been brought to Europe from Anatolia – especially cantaloupes had been taken from Lake Van Basin by Roman missioners. Therefore, there is a wide variation of melon genotype in the basin of Lake Van both due to the reason that melon farming has been carried out since the ancient times and the fertilization biology of the melon.

Arbuscular mycorrhizal fungi (AMF) in the soil are accepted to be one of the most important factors that determine the quality of the soil. There is a great interaction between these fungal symbionts which have key role in rhizosphere and plant which are their hosts and they influence each other in different senses (Duhoux et al., 2001; Kjøller and Rosendahl, 2000, 2001; Janos 2007). Moreover, mycorrhizal dependency of plants influences the population structure and dynamic of plants importantly. In this context, determining AM fungi which are colonized in different plant types and expressing the difference and similarity of these species would be useful in order to carry out plant development both in natural and agricultural ecosystems (Klironomos et al., 2000). In order to do this descriptive procedure faster and safer, it has become compulsory to use molecular techniques. Considering all these facts; part of the studies that have been carried out in recent years have focused on the determination of these rhizosphere elements with molecular techniques (techniques based on PCR) and the relationship between host plant-symbiont (Gardes and Bruns,1993; Van Tuinen et al., 1998

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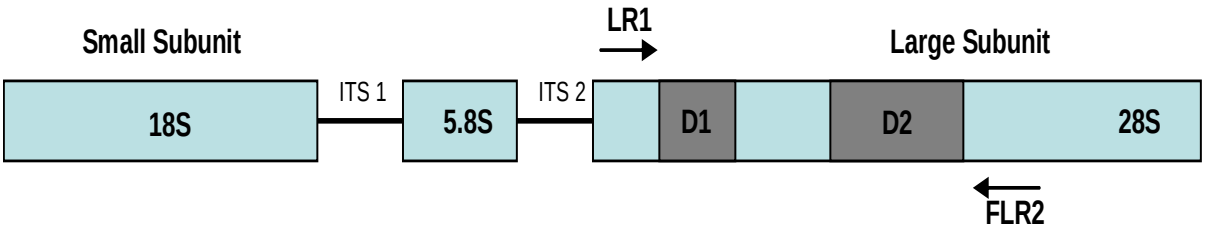

Figure 1. Schematic representation of ribosomal RNA gene and the positions of priming sites.

Jacquot et al., 2000; Kjøller and Rosendahl, 2000; Burleigh, 2001; Alkan et al., 2004; Demir et al., 2011).

This study was aimed to identify melon AMF symbiotic relationship both with classical and molecular methods, detect colonization density and express relationship between melon species and AMF types in basin of Lake Van, one of the gene centers of melon and rich genetic variety area in Turkey.

MATERIALS AND METHODS

Sampling

Survey was conducted in the districts of Van province (Gevas, Ercis, Muradiye and Ercek towns) including the central area. During surveys, considering the fact of influencing possible AMF population negatively, sampling areas which are not intervened chemically were chosen for soil types of melon species.

Soil samples belonging to rizosfer area of melon (*Cucumis melo* L.) were collected at a depth of 0 to 30 cm in late July and beginning of September. Soil samples were collected from the root zones of 100 different plants from 10 locations. In the laboratory, the species of the individual sampled plants were identified. Fungus isolations from soil samples were made using trap plant as corn (*Zea mays* L.). Corn plants were grown in disinfected plastic pots (18 x 18 cm) containing a sterilized mixture of soil and sand (1/1, v/v). Seeds of corn were surface sterilized with procholaraz solution for 30 min (Leopold, 1990) before sowing them into a 5 cm depth of growth media. The plants were grown in a greenhouse under natural photoperiods (23.5/18°C day/night, 4000 to 6000 lux light intensity) for 10 weeks during which only distilled water was applied.

Analysis of root colonization

Melon roots were dyed to detect AMF presence, which was determined using a modification of Phillips and Hayman (1970) method, and the percentage and intensity of mycorrhizal colonization was estimated using the grid line intersect method (Giovanetti and Mosse, 1980). Intraradical colonization and extraradical hyphae development were determined using an intensity rating system for structures (arbuscules, vesicles, internal hyphae and external hyphae), as follows: (0) structures absent; (1) present but scarce; (2) abundant throughout root piece; (3) densely packed throughout root piece (Linderman and Davis, 2004).

Reference AMF species

Four reference AMF isolates, representing two species, were used as positive controls in nested-PCR reactions. They were selected because they were putatively identified in a previous survey of field soils in Van.

DNA preparation

Individual 1 cm long trypan blue stained mychorrhizal root fragments were rinsed in sterile H_2O, crushed in 40 µl TE buffer (10 mM Tris HCl, pH 8 1 mM EDTA) and heated at 100°C for 1 min in the presence of 10 µl 20% Chelex 100 (BioRad). The crude DNA suspension was separated from cellular components by centrifugation at 8.000 rpm for 5 min and 5 µl supernatant was used as target DNA in the first set of amplification (Van Tuinen et al., 1998).

Nested PCR

A two step PCR procedure (nested PCR) was performed as described by Jacquot et al. (2000) with slight modifications. The first step PCR was performed with the eukaryotic-specific primer LR1 (5'-gcatatcaataagcggagga-3') (Van Tuinen et al., 1998) and with the fungal-specific primer FLR2 (5'-gtcgtttaaagccattacgtc-3') (Figure 1) (Trouvelot et al., 1998).

A final volume of 25 µl PCR mixture contained: 2 µl of DNA extract, 2.5 µl of 10X reaction buffer (200 mM Tris-HCL pH: 8.4, 500 mM KCl), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM each), 0.5 µl of each primer (100 pmol/µl), 0.2 µl of *Taq* DNA polymerase and 17.3 µl of RNase free sterile water. The amplifications were performed in a thermal cycler (Eppendorf Mastercycler) programmed as follows: initial denaturation cycle at 95°C (3 min), annealing at 60°C (1 min), extension at 72°C (1 min) followed by 30 cycles of denaturation 93°C (1 min), annealing at 60°C (1 min) and extension at 72°C (1 min); the last cycle was followed by a final extension at 72°C for 5 min. A 5 µl aliquot of this first amplification, diluted 1/100, served as template for a second PCR reaction using taxon-specific primers in combination with FLR2-5.23 (50-gtacggttagtcaacatcg-30) for *Glomus mosseae* (Trouvelot et al., 1998) and FLR2-8.23 (50-gttcggttgatcagatccgct-30) for *Glomus intraradices* (Van Tuinen et al., 1998). Amplification conditions were as mentioned earlier. Aliquots of 10 µl PCR products were separated on 1% agarose gel in TAE buffer (40 mM Tris pH 7.8, 20 mM acetic acid, 2 mM EDTA) and DNA was visualized after ethidium bromide staining (Sambrook et al., 1989).

RESULTS

Determination of AM fungi

Classical method

In the scope of this study, soil samples were gathered from the rhizosphere area of 3 types and 100 melons that are different from each other from 10 locations in total. As a result of the isolation carried out by using trap plant, arbuscular mycorrhizal fungus was detected in 52 melons (Table 1). It was detected that of all the melons in which AM formation was detected, 26 of them belong to

Cantaloupe species, 19 of them belong to *Kirkagac* and 7 belong to *Kirkagac* 637 species. While AMF colonization rates range between 4.5 to 37.8% (Table 1), it was detected that mycorrhizal melon species are located in the heights of 1630 to 1844 m (Table 1). During AMF isolation, identification and determination of colonization rates with classical methods, fungal structures that mycorrhizal fungi form in the roots of trap plants (internal and external hyphae, vesicle, arbuscul and chylamydospores placed inside or outside the root) were considered. All the basic structures of mycorrhizal fungi were examined in the roots of melons in which AM formation was detected. During the detection of AM formation with classical methods, both the species and type identification of fungi were done by using detection keys. However, when observed structures; arbuscule structure, inner and outer spores and wall structure of spores, internal and external hyphae, connection points of hyphae, existence of vesicle are considered, it can be said that *Glomus* type fungi exist as fungal symbiont in all the plant in which mycorrhizal life is observed.

Molecular method

Molecular detection of AM fungi are carried out not from directly rhizosphere soil, but from potting soil prepared from soil of rhizosphere area and diluted with sand and developed by using trap plants (corn, leek, marigold, etc.), due to the difficulty of purification of AMF spores (Kjøller and Rosendahl, 2001; Van Tuinen et al., 1998). In this study, molecular detection of SM fungi were done not by using directly rhizosphere soil, but by using corn as trap plant and with DNA extraction from plant roots and nested-PRC technique following it.

Originally, the extraction method as defined by van Tuinen et al. (1998) was used to extract DNA from root samples. The method yielded enough PCR products to use as positive control. Upon sequential amplification with the LR1/FLR2 and FLR2/8.23 (5'-gttcggttgatcagatccgct-3') and FLR2/5.23 (5'-gtacggttagtcaacatc G-3') primer pairs, for the detection of *G. intraradices* and *G. mosseae* the PCR products of the expected size (574 and 264 bp, respectively) were observed for all the reference isolates tested.

Four of the 100 samples processed had prominent PCR bands of *G. intraradices* and seven of *G. mosseae* (that is, bands within mobility range of reference bands) (Figures 2 and 3). The presence of both AMF species was confirmed in the rhizosphere soil of the surveyed melon fields (Table 2). In approximately 21.15% of 52 melons in which AMF formation was detected with classical methods; arbuscular mycorrhizal fungi could be detected in the basis of species molecularly.

DISCUSSION

One of the main purposes of this study is to detect mycorrhizal formation of melons and the other is to do the detection of these fungi with the help of molecular methods. Therefore, during the detection of AM formation with classical methods, both the species and type identification of fungi were not done by using detection keys. However, when observed structures are consi-dered, it can be said that *Glomus* type fungi exist as fungal symbiont in all the plant in which mycorrhizal life is observed. Schenck and Smith (1982) and Morton and Bentivenga (1994) indicated that *Glomus* species are the most common AM fungi in the sense of propagation on earth and that *G. mosseae*, *G. intraradices* and *Glomus occultum* are the species which have the highest aggres-sivity. In this study, 11 melons (21.15%) in which AMF formation was detected were determined molecularly as a result of nested-PCR practices and 7 of these fungi (63.6%) are of *G. mosseae* species and 4 of them (36.4%) are of *G. intraradices* species. In many studies concerning the detection of AMF fungi both with PCR and nested-PCR techniques, it was detected that fungi of *Glomus* genus are determined and they are generally of *G. intraradices*, *G. mosseae*, *Glomus versiforme* ve *Glomus caledonium* species (Jacquot et al., 2000; Redecker, 2000; Renker et al., 2003; Stukenbrock and Rosendahl, 2005).

In classical methods, it was detected that there is a mycorrhizal life in nearly 52% of total plant number and in nearly 21.15% of this rate AM fungi could be detected in the level of species molecularly. When the numerical parameter is considered, it can be said that among 52 plant which have arbuscular mycorrhizal life, fungi in 11 of them are detected molecularly. Stukenbrock and Rosendahl (2005) stated that of all the 812 root samples; 186 of them respond nested-PCR positively and that this value corresponds to 22.7% in total.

As a result, as it is mentioned in this study, since studies concerning identification and detection, molecular techniques based on PCR (conventional PCR, nested PCR, real-time Scorpion PCR, etc.) enable fast charac-terization and faster and safer identification of soil types, they draw great interest in the detection of AM fungi.
Another point that draws attention in addition to this is that the study was carried out in Van vicinity which is regarded as the second most important gene center of melon. It is an indispensable truth that variation of AMF accommodation is similarly high in melon which is also rich in the sense of genetic variation. Considering this fact, some of the AMF isolates which was detected as a result of the study and whose efficiency will be detected in further studies are regarded to be quite useful in the practice of melon farming considering the species-AMF accommodation.

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Table 1. Melon species colonized by AM fungi, intensity rating system for structures, colonization rates of mycorrhizal fungi, GPS and altitude (m) values belong melon species determined arbuscular mycorrhizal formation in Van and its districts.

Plant code	Species of melon	Intensity rating system for structure*	Colonization (%)	GPS value	Altitude (m)
ERGA1	Kirkagac	1	10.5	43°22′12″ 38°59′57″	1661
ERGA2	Kirkagac	1	17.2	43°22′12″ 38°58′52″	1663
ERGA4	Kirkagac	1	16.8	43°22′11″ 38°58′59″	1660
ERGA6	Kirkagac	2	22.6	43°22′10″ 38°58′56″	1630
ERGA8	Kirkagac	1	14.2	43°23′10″ 38°58′58″	1658
ERGA9	Kirkagac	1	18.6	43°22′10″ 38°58′59″	1652
ERP4	Kirkagac	1	8.6	43°21′41″ 38°59′12″	1662
ERP6	Cantaloupe	1	21.8	43°21′42″ 38°59′10″	1667
ERP7	Cantaloupe	2	16.8	43°21′43″ 38°59′09″	1675
ERP11	Cantaloupe	1	12.4	43°21′41″ 38°59′09″	1676
ERIN1	Cantaloupe	1	14.4	43°28′26″ 39°59′21″	1686
ERIN3	Cantaloupe	1	11.8	43°28′26″ 39°59′22″	1676
ERAG1	Cantaloupe	2	28.5	43°29′19″ 39°59′22″	1683
ERAG2	Cantaloupe	1	16.6	43°29′19″ 39°59′22″	1684
ERAG3	Cantaloupe	2	23.0	43°29′19″ 39°59′22″	1685
ERAG4	Cantaloupe	1	8.3	43°29′21″ 39°59′22″	1686
ERAG5	Cantaloupe	1	5.5	43°30′40″ 38°59′29″	1687

Table 1. Continue.

ERU1	Kirkagac	1	8.0	43°30′40″ 38°59′27″	1665
ERU3	Kirlagac 637	2	26.8	43°30′40″ 38°59′26″	1662
ERU4	Kirkagac 637	2	36.2	43°30′40″ 38°59′26″	1659
ERU5	Kirkagac 637	2	24.4	43°30′40″ 38°59′26″	1662
ERPGA3	Kirkagac 637	1	11.0	43°21′41″ 38°59′14″	1669
ERPGA4	Kirkagac 637	1	8.3	43°21′40″ 38°59′14″	1667
ERPGA5	Kirkagac 637	1	9.5	43°21′39″ 38°59′14″	1666
ERPGA7	Kirkagac 637	1	11.7	43°21′41″ 38°59′14″	1669
ERA1	Kirkagac	1	18.4	43°31′13″ 38°59′24″	1672
ERA2	Kirkagac	1	16.4	43°31′13″ 38°59′24″	1675
ERA3	Kirkagac	1	14.6	43°31′14″ 38°59′26″	1676
ERA5	Kirkagac	1	13.4	43°31′15″ 38°59′27″	1679
ERI1	Cantaloupe	1	8.6	43°21′48″ 38°59′24″	1668
ERI2	Cantaloupe	1	21.7	43°21′48″ 38°59′24″	1671
ERI3	Cantaloupe	1	8.3	43°21′48″ 38°59′23″	1676
ERI4	Cantaloupe	2	27.6	43°21′48″ 38°59′24″	1678
ERI8	Cantaloupe	1	15.4	43°21′41″ 38°59′22″	1661
ERI10	Cantaloupe	1	13.0	43°45′49″ 38°56′23″	1666

Table 1. Continue.

MY1	Kirkagac	2	25.6	43°45′48″ 38°56′22″	1698
MY4	Kirkagac	1	9.4	43°45′48″ 38°56′23″	1692
MY5	Kirkagac	2	21.6	43°45′48″ 38°56′21″	1703
MY7	Kirkagac	1	12.8	43°45′48″ 38°56′23″	1702
MY8	Kirkagac	2	32.6	43°45′47″ 38°56′24″	1703
MY9	Kirkagac	1	16.8	43°45′48″ 38°56′24″	1701
MY13	Kirkagac	1	7.8	43°45′48″ 38°56′25″	1709
ERC6	Cantaloupe	1	9.5	43°35′41″ 38°36′59″	1839
ERC7	Cantaloupe	1	4.5	43°35′41″ 38°36′58″	1832
ERC8	Cantaloupe	2	31.8	43°35′41″ 38°36′58″	1844
ERC9	Cantaloupe	2	26.0	43°35′41″ 38°36′58″	1842
ERC11	Cantaloupe	1	14.6	43°35′41″ 38°36′58″	1844
ERC14	Cantaloupe	1	8.2	43°35′41″ 38°37′58″	1834
ERC17	Cantaloupe	1	13.4	43°35′50″ 38°37′59″	1824
ERC19	Cantaloupe	1	15.6	43°38′41″ 38°59′58″	1841
ERC22	Cantaloupe	2	37.8	43°35′41″ 38°36′58″	1843
ERC25	Cantaloupe	2	23.6	43°35′41″ 38°36′58″	1828

Intensity rating systemfor structures (arbuscules, vesicles, internal hyphae, external hyphae), as follows: (0) structures absent; (1) present but scarce; (2) abundant throughout root piece; (3) densely packed throughout root piece.

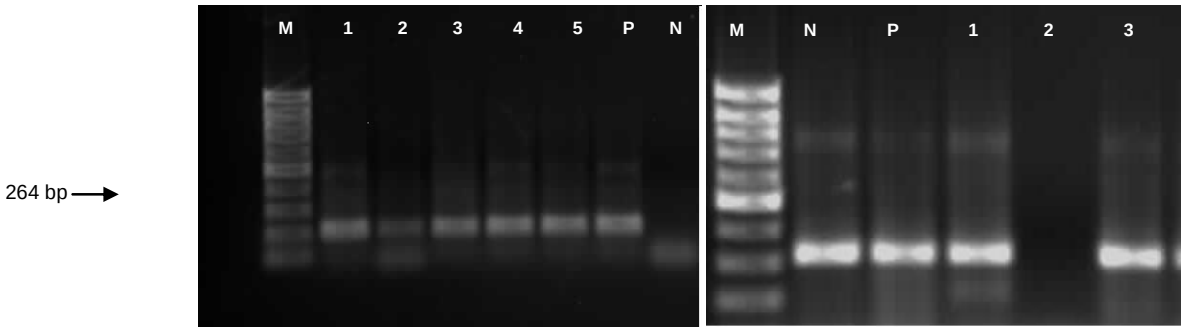

Figure 2. Detection of *G. mosseae* by nested-PCR in root samples of melon species. M: DNA ladder, P. positive control, N: negative control, 1-5 and 1-3 tested samples.

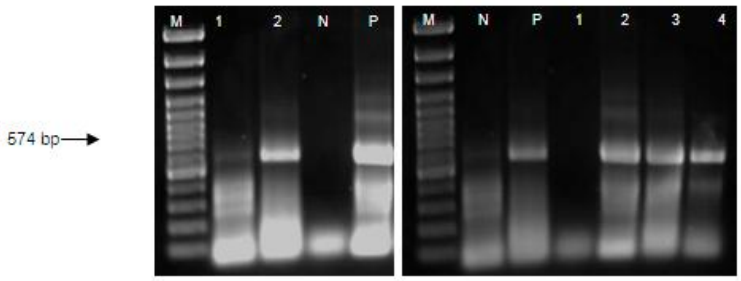

Figure 3. Detection of *G. intraradices* by nested-PCR in root samples of melon species. M: DNA ladder, P. positive control, N: negative control, 1-2 and 1-4 tested samples.

Table 2. Species of melon determined mycorrhizal formation and AM fungi by nested-PCR.

Plant code	Species of melon	Speceis of AMF
ERÜ1	Kiragac 637	*Glomus intraradices*
MY5	Kirkagac	*Glomus intraradices*
ERİ4	Kantalop	*Glomus intraradices*
ERPGA4	Kiragac 637	*Glomus intraradices*
ERPGA5	Kiragac 637	*Glomus mosseae*
MY8	Kirkagac	*Glomus mosseae*
MY4	Kirkagac	*Glomus mosseae*
ERGA6	Kirkagac	*Glomus mosseae*
ERİN1	Kantalop	*Glomus mosseae*
ERİN3	Kantalop	*Glomus mosseae*
ERİ10	Kantalop	*Glomus mosseae*

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