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A Novel Technique for The Recovery, Isolation and Preliminary Evaluation of *Rhizoctonia solani* Mycoparasites from Soil

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ABSTRACT

This paper describes a novel technique that allows detecting and isolating of *R. solani* mycoparasites subsist in soil incorporating the precolonized agar method and a modified nutrient medium supplemented with tolclophos-methyl. A great number of potential biocontrol agents like *Trichoderma* and *Gliocladium* could be isolated into pure culture. Most of the isolates examined macroscopically and microscopically in dual cultures had been recognized as mycoparasites. By using this novel technique, various mycoparasitic isolates were obtained from soil. The majority of the isolates were in the genera of *Trichoderma* and *Gliocladium*. Fourteen *Trichoderma* species, *Gliocladium roseum* Bainier, *Chaetomium* sp., *Geotrichum* sp., *Paecilomyces* sp., *Papulospora* sp. and a *Spicaria* sp. were determined as having mycoparasitic activity against *R. solani* as well. Some of them possessed both parasitic action and antifungal antibiotic activity. This novel technique proved to be useful and showed promise for studies of the ecology of biocontrol agents added to soil or seed.

Keywords: Isolation technique, mycoparasite, *Rhizoctonia solani, Trichoderma, Gliocladium,* tolclofos methyl

Topraktaki *Rhizoctonia solani* Mikoparazitlerinin Saptanması, İzolasyonu ve Ön Değerlendirilmesi İçin Yeni Bir Yöntem

ÖZET

Bu yayın, toprakta bulunan *R. solani* mikoparazitlerini saptamak ve izole etmek amacıyla "petri kabında agarlı ortamda geliştirilmiş *R. solani* kolonisi üzerine toprak örneği ekleyip bir süre beklemek ve bundan cork-borer ile kesilen parçaları tolclophos-methyl içeren özel besiyerine aktarmak" şeklinde özetlenebilen yeni bir yöntemi açıklamaktadır. Bu yöntemi kullanarak, *Trichoderma* ve *Gliocladium* gibi potansiyel biyokontrol etmenlerinden birçok saf kültür elde edilebilmiştir. İkili kültürlerde makroskopik ve mikroskobik olarak incelenen izolatların çoğunun mikoparazit olduğu anlaşılmıştır. Bu yeni tekniği kullanarak topraktan farklı mikoparazitik izolatlar elde edilmiştir. İzolatların çoğunluğu *Trichoderma* ve *Gliocladium* cinslerine aittir. Ondört farklı *Trichoderma* türü, *Gliocladium roseum* Bainier, *Chaetomium* sp., *Geotrichum* sp., *Paecilomyces* sp., *Papulospora* sp. ve *Spicaria* sp.'nin de *R. solani'* ye karşı mikoparazitik aktiviteye sahip olduğu saptanmıştır. İzolatlardan bazılarının hem parazitik hem de antifungal antibiyotik etkili olduğu anlaşılmıştır. Bu yeni yöntemin toprağa veya tohuma uygulanan biyokontrol etmenlerinin ekolojisi konusundaki çalışmalarda yararlı olacağı öngörülmüştür.

Anahtar Kelimeler: İzolasyon yöntemi, mikoparazit, *Rhizoctonia solani, Trichoderma, Gliocladium,* tolclofos methyl

INTRODUCTION

There is an urgent need for new technologies of crop protection. Traditional methods used to protect crops from diseases have been largely based on the use of chemical pesticides. But today there are strict regulations on

chemical fungicide use due to carcinogenic effects, residual toxicity problems, environmental pollution and development of fungicide resistant strains but also strong political and public pressure to remove the most hazardous chemicals from the market. Therefore many researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases and different strategies have been hypothesized (Pal and Gardener, 2006; Vinale et al., 2008; Abo-Elyousr et al., 2014).

One of the most promising means is the use of new tools based on biocontrol agents. The use of antagonist microorganisms against fungal plant pathogens is an attractive and environmentally-friendly alternative to the use of chemical pesticides. Control of fungal plant diseases using naturally occurring non-pathogenic microorganisms represents a promising approach for the control of plant disease. Biological control using antagonistic microbes alone, or as supplements to minimize the use of chemical pesticides in an integrated plant disease management system, has become more prevalent in recent years.

Rhizoctonia solani Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is one of the most important, universal, destructive soilborne phytopathogenic fungi that causes various diseases in many plant species world-wide. It is responsible for the damping-off on seedlings and root rot, stem rot, stem canker, foliage blights or spots near the ground and rotting of storage organs (Banville et al., 1996; Boosalis and Scharen, 1959).

Mycoparasites and their possible use in biological control have been recognized since 1932. Since that time there are a number of examples of fungi that parasitize plant pathogens including *R. solani*. Considerable work has focused on various *Trichoderma* and *Gliocladium* species; they have been studied to the greatest extent and possess great potential as biocontrol agents (Weindling, 1932; Barnett and Binder, 1973 ; Lumsden, 1981; Elad et al., 1980, 1983; Papavizas, 1985; Adams, 1990; Coley-Smith et al., 1991; Wilson et al., 1992; Cuevas et al., 1995; Lumsden et al., 1995; Hjeljord and Tronsmo, 1998; Kredics et al., 2003; Samuels, 2006; Aydın and Turhan, 2013).

Trichoderma spp. are basically soil-borne saprophytic fungi which possess a symbiotic relationship with the plant rhizosphere. They are well known for their antagonism against several fungi, to produce different antibiotic substances and to inhibit the growth of pathogenic fungi by modifying the rhizosphere. The antagonistic properties of *Trichoderma* spp. towards fungal pathogens are based on strong mycoparasitism, antibiosis, competition for nutrients and space, promotion of plant growth and plant defense responses. They are widely used as commercial biofungicides all over the world (Dennis and Webster, 1971; Chet and Baker, 1981; Chet et al., 1981; Howell, 2003, 2006; Benítez et al., 2004; Harman et al., 2004; Harman, 2006; Almeida et al., 2007; Verma et al., 2007; Woo and Lorito, 2007; Bailey et al., 2008; Vinale et al., 2008; Amin et al., 2010; Anees et al., 2010; Brotman et al., 2010; Chaverri et al., 2015; Hermosa et al., 2012; Kumar et al., 2012; Hyakumachi, 2013; Abo-Elyousr et al., 2014; Asad et al., 2014; Mukherjee et al., 2014; Naher et al., 2014; Rinu et al., 2014).

The isolation of *Trichoderma* and *Gliocladium* from soil *was* investigated frequently by researchers using a variety of methods such as “serial dilution plate technique”, “ pour plate”, “differential centrifugation technique”, “the slide-trap method” and “the dilution plate count technique” (Hopkins et al., 1991; Aydın and Turhan, 2009; Vargas Gil et al. 2009).

Furthermore, several authors have reported the use of agar cultures of fungi or their resting bodies like sclerotia as selective bait for the isolation of specific mycoparasites from soil or from natural habitats. It is possible to specifically select the mycoparasitic fungi by using agar plate precolonized with a suitable host fungus as a bait. Promising results have been obtained using this method and various mycoparasitic fungi including *Trichoderma* spp., *Gliocladium* spp. and others could be isolated (Deacon and Henry, 1978; Foley and Deacon, 1985; van den Boogert and Gams, 1988; Lodha and Webster, 1990; van den Boogert et al., 1990; Deacon and Berry, 1992; Mulligan and Deacon, 1992; Mulligan et al., 1995; Knudsen et al., 1997; Crauss et al., 1998; Molan, 2009).

Ribeiro and Butler (1992) used the “sclerotia bait technique” for the isolation of *Pythium* species mycoparasitic on *Sclerotinia sclerotiorum. Trichoderma* strains were isolated from *S. sclerotiorum* sclerotia by selective isolation employing the sclerotial bait technique (Melo et al., 1996). Ali-Shtayeh and Saleh (1999)

reported that the sclerotia baiting technique was highly effective for the detection and isolation of some mycoparasitic *Pythium* species from soils assayed. Baiting and filtering techniques are used to recover species of *Phytophthora* and *Pythium* from water and soil (Roberts et al., 2005). Karaca et al. (2008) used the sclerotia of *Botrytis cinerea* and *S. sclerotiorum* as baits for the isolation of the mycoparasitic fungi from Oomycetes. Burgess and Hepworth (1996) used sclerotia of *S. minor* as baits to isolate *T. virens* from soil. Selective isolation of mycoparasites of *S. minor* from soil by baiting proved to be useful for enumerating *T. virens* on roots of sunflower and showed promise for studies of the ecology of biocontrol agents added to soil or seed (Isnaini et al., 1998). Human hair baiting technique was followed to isolate dermatophytes and related keratinophilic fungi from the soil samples (Kachuei et al., 2012; Malek et al, 2013; Deshmukh and Verekar, 2014). The larvae of *Galleria mellonella*, were used as a standard bait insect for the isolation of entomopathogenic fungi, because the larvae are highly susceptible to infection by these fungi (Chandler et al. 1997; Meyling, 2007; Goble et al., 2010, 2012; Hernández-Domínguez et al., 2016).

The objective of this study was to improve the isolation efficiency of mycoparasitic fungi, especially *Trichoderma* spp. and *Gliocladium* spp. from soil applying precolonized *R. solani* culture as a trap in the first stage and using a modified nutrient medium supplemented with tolclophos-methyl in the second.

MATERIALS AND METHODS

The isolation process of *R. solani* mycoparasites

For the isolation of *Rhizoctonia* mycoparasites, the soil samples were taken from 11 different ecological habitat in Turkey. They were collected from a depth of 1-15 cm, litter-free layer; transported to the laboratory in plastic bags and processed immediately. An isolate of *R. solani* recovered from diseased potato roots was used for all experiments. First, *R.solani* was allowed to cover the PDA plate completely. Then somewhat soil was placed onto the precolonized plate and incubated in the dark at 24 °C for two weeks. After removal of the soil particles, the underlying *Rhizoctonia* colony was thoroughly washed with sterilized water. Potato Dextrose Agar (Merck 1.10130) was sterilized in autoclave after adding 33 mg/L Rose Bengal (BDH Chemicals). Than Streptomycin sulfate (Sigma, 200 mg/L) dissolved in 10 ml sterile distilled water and tolclofos-methyl (Rizolex®, Sumimoto, 12 mg/L) dissolved in 10 ml 95% ethyl alcohol were added after the sterile medium had cooled to 50°C. Discs (5 mm) cut from the freshly washed *R. solani* colony were transplanted (mycelial side beneath) onto the modified PDA containing rose bengal, streptomycine and tolclophos-methyl (33 mg/L, 200 mg/L and 6 mg active ingredient/L, respectively) and reincubated approximately for two weeks on a laboratory bench at room temperature (Figure 1, 2). Variegated fungi grown in the meanwhile were isolated into pure cultures and evaluated then for their potential to parasitize *R.solani* in dual cultures.

Demonstrating the mycoparasitic nature of the isolates

A mycelial disc (5 mm) of the potential mycoparasitic isolate was placed onto the surface of modified PDA (ten times diluted PD broth + 15 g/l agar). According to growth rate of candidate antagonist, either simultaneously or two days later, a mycelial disc of *R. solani* was placed 4 cm apart onto the same plate. The mycelial disc of pathogen or antagonist grown alone served as control. Then the plates were incubated in the dark at 24 °C. As from the paired colonies reached each others after some days, overgrowth of *R. solani* by mycoparasitic isolate could be detected. Directly interaction area or only mycelial samples taken from there were examined under light microscope. Throughout the experiments, there were three replications.

RESULTS AND DISCUSSION

When the colonies grown in dual cultures come into contact, the mycoparasite has continued to grow rapidly through the *Rhizoctonia* colonies. When cultures intermingled, the physical interaction between the mycoparasites and *R.solani* was examined by light microscopy. Hyphae of *R. solani* and hyperparasites were distinguished from each other by the orientation of hyphal growth. The fine hyphae of the mycoparasites were also readily

differentiated from the coarse hyphae of *Rhizoctonia*. Tight coiling by the mycoparasites around *Rhizoctonia* hyphae was observed. The internal parasitism was evidenced by the fact that parasitic hyphae were frequently found inside the host hyphae

Figure 1. Mature *Rhizoctonia solani* colony on PDA (a), waiting period as covered with soil sample (b)

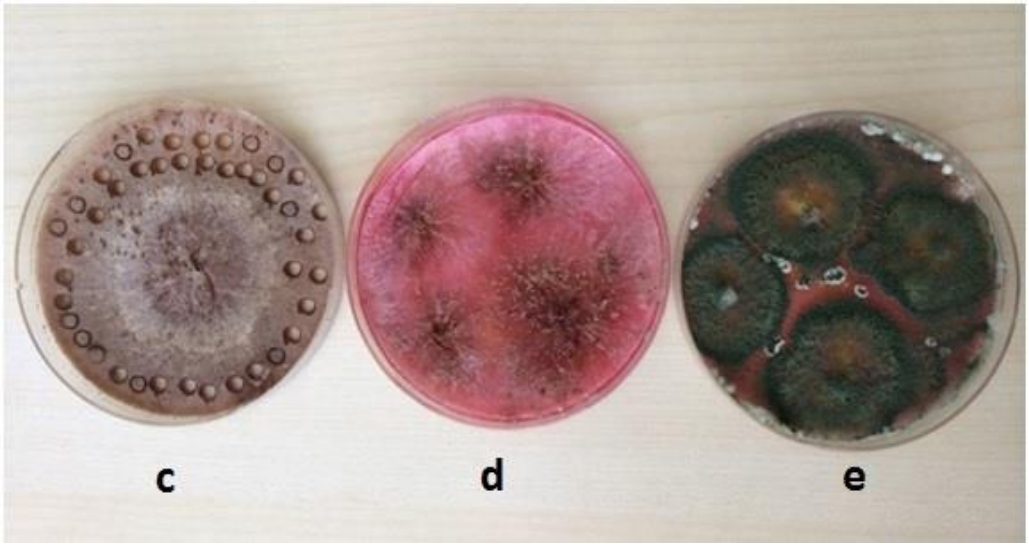

Figure 2. Discs cut from thoroughly washed colony (c), rapid growth of *Rhizoctonia solani* alone on PDA having Rose Bengal and Streptomycine (d), growth of mycoparasites on the same medium having additionally tolclophos-methyl (e).

By using the novel technique introduced in this study, 264 mycoparasitic isolates were obtained from soil. The majority of the isolates were found to be in the genera of *Trichoderma* and *Gliocladium*. Fourteen *Trichoderma* species, *Gliocladium roseum* Bainier, *Chaetomium* sp., *Geotrichum* sp., *Paecilomyces* sp., *Papulospora* sp. and a *Spicaria* sp. were determined as having mycoparasitic activity against *R. solani* as well. Identification of mycoparasites made by Prof. Dr. W. Gams, Prof. Dr. G. J. Samuels and Prof. Dr. G. Turhan.

Mycoparasitism, the direct attack of one fungus to another, is a very complex process. It has been known for many years that the genus *Trichoderma* has a strong mycoparasitic activity; it can sense the presence of target fungi;

it is attracted to grows towards its host and appeared to grow typically, probably by chemotropism toward them (Chet et al., 1981; Hyakumachi, 2013).

Trichoderma harzianum, T. viride, T. pseudokoningii, T. hamatum, T. virens, T. koningii, T. aureoviride and *Trichoderma* spp. were previously isolated by using known conventional methods in Turkey (Turhan, 1973; İren et al., 1988; Çeliker and Nemli, 1994).

It is possible to specifically select the mycoparasitic fungi by using precolonized agar plate with the host fungus. Only fungi that are able to derive nutrients from the precolonizing fungus and to tolerate its waste products are able to grow. Unfortunately there is no universally susceptible host fungus that can be used in this technique. Since all mycoparasitic fungi have restricted host ranges, the host fungus used to precolonize the plate will greatly influence the type of mycoparasite that will be detected. Mycoparasites isolated by this method include *Gliocladium roseum* and related species (Foley and Deacon, 1985), *Papulaspora* sp. (Deacon and Berry, 1992; Mulligan and Deacon, 1992), *Pythium oligandrum* and related species (Deacon and Henry, 1978; Foley and Deacon, 1985; Mulligan et al., 1995) and *Verticillium biguttatum* (van den Boogert and Gams, 1988).

In the present study the precolonized plate method was used at the first stage of the technique. But, because of the extremely fast growing rate of *R. solani*, it was difficult to catch it by invaders, even though it is already parasitized during the waiting period beneath the soil coating in petri dishes. Hence *R. solani* grew first from the precolonized discs and completely filled the plate in a little while (Figure 2d) unless the presence an intentional inhibitive in the culture medium.

The results of a study by Hameed (2008) showed that Rizolex® (50% Tolclofos methyl) was effective on radial growth of *R. solani.* There were differences between *R.solani* and *T.harzianum* in response to Rizolex, then isolates of *R.solani* were inhibited completely at all concentrations tested, while *T.harzianum* was affected lesser. Yobo et al.(2010) found that although *R. solani* growth was completely inhibited in concentrations of 0.05 and 0.125 g ai/l tolclofos-methyl, *Trichoderma* isolates tested were less sensitive to this chemical.

We hypothesized that *R.solani* is more susceptible to tolclophos-methyl than *Trichoderma* and *Gliocladium* spp. Based on this hypothesis, we used a special growth medium supplemented with tolclofos methyl. The purpose of adding this chemical into the isolation medium was to prevent extreme speedy growth of *R. solani*, which already existed on precolonized discs. Really, the presence of tolclophos-methyl in nutrient medium prevented the unwelcome growth of *R. solani* significantly. Fortunately, Trichoderma and Gliocladium isolates were least affected displaying considerable tolerance to this fungicide and they could grow well on this modified PDA medium.

On account of the lesser sensitivity of two foremost important mycoparasitic genera to tolclophos-methyl than *R. solani*, it can be concluded that this chemical could be used in integrated management in addition to biocontrol agents against this pathogen.

Furthermore, the technique described, without doubt, may also be used for detecting and isolating the mycoparasites of other fast growing fungus and fungus-like organisms such as *Pythium* from soil by changing the supplemented chemical which suppress only the target pathogen.

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