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INDUCED *IN VITRO* CO-CULTURE BETWEEN *ASPARAGUS OFFICINALIS* L. PHYTOINOCULA AND A SAPROPHYTIC FUNGUS

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ABSTRACT.
The aim of present research is to investigate the nature of the relationship established among partners, forced associates, depending on ecophysiological conditions, namely induced *in vitro* co-culture between *Cladosporium* sp. fungus and *Asparagus officinalis* L. phytoinocula. At 30 days from co-culture initiation, on Murashige-Skoog mineral medium, without growth regulators, lightened with white fluorescent light, 1700 lx intensity, with the photoperiod of 16 h day length/24h, and a temperature of 20 ± 2°C, the two partners no interacted, and both had normally developed. *Asparagus* explants provided from a co-culture with *Cladosporium* sp. which was individual cultivated on fresh medium, ulterior showed no infection.
Keywors: fungus, *Asparagus*, co-culture, micropropagation

INTRODUCTION

Plant biotechnology will be the domain that, in the future, will provide much needed human nutrition, in addition to traditional methods (Antofie et al., 2010 a and b). *In vitro* cultures are used for many plant species, which enables us to obtain healthy plants in a short time. In practice there are, however many difficulties connected with the obtaining of regenerative active and viruses - free explants (Cassells, 1991; Cooper et al., 2006).

One of the problems is the infection of phytoinocula caused by fungi, drawing into the elimination of explants from further micropropagation techniques. The results of works done so far Leifert and collaborators (1994), Debergh (1996), Leifert and Woodward (1997), Williamson and collaborators (1997) present a wide variety of fungi species, appearing in the *in vitro* cultures, on the subsequent stages of micropropagation.

About thirty-one microorganisms from ten different plant cultivars growing in micropropagation have been isolated, identified and characterized, with Yeasts, *Corynebacterium* spp. and *Pseudomonas* spp. being predominant (Leggatt et al., 1994).

On the other hand, in the present, in modern plant biotechnology, there is a new scientific branch, a "hybrid" between biotechnology focused on phytoinocula and that focusing on fungi, namely the *co-culture* (associated culture) (Cachiţă et al., 2008; Turcuş and Cachiţă, 2009), purpose of establishing symbiosis, raising productivity of plants, especially fruit and forest species or increase the synthesis of secondary metabolites, or the selection of plants to certain pathotoxin resisted etc. (Cachiţă and Ardelean, 2009).

The tissue cultures can be used for studying host-pathogen interaction (Ingram and Helgeson, 1980; Devi and Srinivasan, 2006), supplementing the screening field, helping to accelerate the assessments and studies in this area. This method have more advantages: using of sterile host in aseptically conditions; small tissue used to reduce the space needed; the size of inocula, the pathogen penetration mode and rapid assessment can be done, deliberate manipulation of physical and chemical environment and no unnecessary risk of introduction the pathogen to the field (Nik and Rahman, 1993).

In natural conditions, root rot in *Asparagus* caused by two species of *Fusarium* (*Fusarium oxysporum* f.sp. *asparagi* and *Fusarium proliferatum)* is present in many cultures (Lewis and Shoemaker, 1964; Dan and Stephens, 1995), being the major cause of asparagus disease with great loses in commercial planting of asparagus and other varieties of *Fusarium oxysporum* affect cabbage, radish, carnation, cotton, melon, potato and tomato (Davis, 1969), *Pinus resinosa* (Farquar and Peterson, 1989) or banana (Matsumoto et al, 2010). This fungus produced toxins like fusaric acid (FA), lycomarasmin, moniliformin, naphthazarin, sambucin and beauvericin (Švábová and Lebeda, 2005) and the chemical treatment is no effective. Cassini et al. (1985) employed an antagonist microbe to control *Fusarium* infections of field-grow asparagus.

There are antifungal antibiotics from actinomycete, like faerie fungi which were used in asparagus culture, without adversely affect (Smith et al., 1990). This antibiotic at 25 ppm decreased the severity of disease in sterile asparagus plant grown on water agar and increased fibrous root weight of plants grown in the absence of *F. oxysporum.* The producer organism, grown on several media, found to be antagonistic to *F. oxysporum.* In the other hand, many papers and reviews have discussed the advantages of using chitinases for plant protection because these enzymes are fungicidal, part of the plant defense system, and nontoxic to plants, but *Cladosporium fulvum*, *Cercospora nicotiana*, *C. beticola*, *Fusarium solani*, and several other pathogenic fungi were not inhibited in the

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growth by treatment with a enzyme namely chitinases obtained from plant (Joosten et al., 1995).

Also, genus of *Asparagus* can be infected by *Stemphylium botriosum* a fungus which affect cladodes, branches and the main stem, with defoliation (Bansal et al, 1986) or by *Puccinia asparagi* that causes rust on *Asparagus*, or *Phytophtora* ssp., *Pleospora herbarum* (Koike et al., 2007).

Rahman and collaborators (1987) used the *in vitro* method on conifer adventition shoots inoculated with *Gremmeniella abietina* to elucidate host-pathogen interaction.

These studies come to indicate a possible positive effect could be having the contamination of *in vitro* plant cultures with a saprophytic fungus.

MATERIAL AND METHODS

Asparagus minicuttings from the greenhouse, with 3 cm length was surface sterilized with 4 % sodium hypochlorite (v/v). Then the fragments were rinsed 5 x with sterile water for 5 minutes each. After necrosis removal 1 cm fragments, with nodes, was placed on solidified Murashige-Skoog (MS) (1962) medium, without growth regulators or aminoacids, with 20 g/l sucrose. The culture was incubated at 22 - 23°C, at 1700 lux light intensity, generated by white fluorescent lamps, with 16/24 light photoperiod, during 8 weeks. After that, the 3 cm long plantlets were transferred to fresh same MS media, kept *in vitro* for other 12 weeks.

The number of culture recipients was 400. Just in one recipient, at 6 week after inoculations, the spontaneous infection appeared. The infection increased, the fungus developed a like-velvet mycelium, with gray colour, whose hyphae no invading culture medium deep, and in 12 weeks had a fructification body (Fig. 1), looking like a crater, about 1.5 cm height, with a wall about 3 mm, with dense structure of hyphae and numerous spores. In other instances, a fungal infection of such scale could be smothered phytoinocula. Paradoxically, not revealed any incompatibility between fungus and phytoinocula, both have a long extending, without interacting, so that at 6 weeks after initial spontaneous co-culture (12 week after inoculation) we proceed to subculture after protocols from Table 1:

Table 1.

The protocol of second induced co-culture.

Type of phytoinocula	Culture media	Culture period
CC-*Asparagus* shoots (1 cm length) and fungus mycelium (0.5/0.5 cm)	MS solidified, without growth regulators	30 days
AC-*Asparagus* shoots (1 cm length)		
FC-Fungus mycelium (0.5/0.5 cm)		

Note: CC – co-culture; AC – *Asparagus* culture; FC – fungus culture.

This subculture was made in culture vessel with 2 cm diameter and 7 cm height.

Assessment was carried out as 30 days after second co-culture initiation.

From fructification body was prelevated fragment which was observed at optical microscope Leitz brand, Webster M and the most representative images were taken with an adapted digital camera.

RESULTS AND DISCUSSIONS

Fungus identified by us is identical to that described by Cachiță and collaborators (2008 a and b), respectively one species of the genus *Cladosporium*. If at that time, the above mentioned authors stated that at *Drosera rotundifolia* L. was highlighted this allelopathy lack of response, explained that this is a carnivorous plant, in these studies can prove that the species of the genus *Asparagus* (monocotyledonous plants) have the same co-culture tolerance with a fungus.

Since, the occurrence of the infection (Fig. 1 A and B) was after the discharge a considerable time after *in vitro* culture initiation, we considered that the cause of this phenomenon was polyethylene foil with which we covered the culture vessel. This hypothesis was rejected by a test which used a lot of vitroculture which was covered with this type of film, and another identical blocked by aluminium foil (which were initially autoclaved the vessel, so it was sterilized). At 6 weeks after inoculation in these conditions of covering, infection occurred in both groups. Other possible causes of infection occurrence may be: improper sterilization of culture medium, culture vessel, instrumentation necessary for inoculation or a error in technique, but in all these cases, the occurrence of infection should be in the first 5-7 days after inoculation. Another hypothesis would be the existence of an ill plant material in earlier vitroculture, but it is not supported by the fact that, from a bush located in vitroculture, the propagules was multiplicated in 10 new vessels, and our new infection was singular. Probably, the fungus spores are in the growth room and penetrate the polyethylene film in its sensitive areas. Such a situation has worried us very much, because there is a risk of a pandemic.

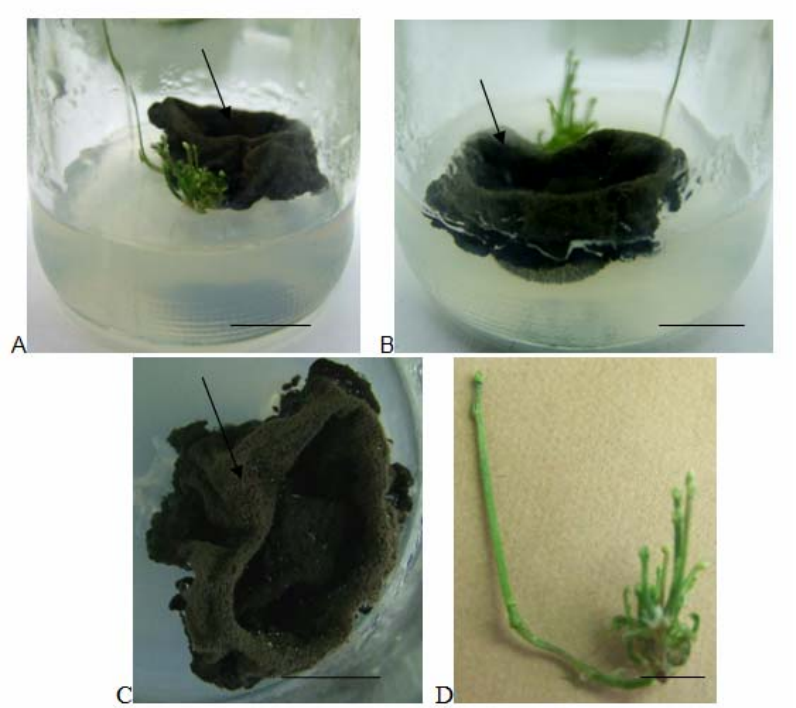

Fig. 1. Spontaneous appearance of *Cladosporium* sp. fungus in *Asparagus officinalis* L. vitroculture, after 12 week of inoculation: A, B – *in vitro* co-culture between phytoinocula and fungus, C- wall of fructification body detail, D – *Asparagus* vitroplantlets provided from spontaneous co-culture (arrow means the fungus fructification body; bars means 1 cm) (original).

Odutayo and collaborators (2007) investigated the source of microbial contamination in tissue culture laboratories, in south-western Nigeria. They observed nineteen microbial contaminants (consisting of eleven bacteria and eight fungi) were found associated with the tissue culture plants and the laboratory environments. The rate of occurrence of *Streptococcus aureus, Bacillus cereus*, *B. subtilis* and *Escherichia coli* were found to be higher (ranging from 36-46%) in human skin than in all other sampled materials. The laboratory walls and tables also harboured most of the contaminating microbes. The laboratory indoor air was found associated with all the contaminating microbes.

Omamor and co-workers (2007) identified twenty-five species of fungi belonging to 14 genera as fungal contaminants of the oil palm tissue culture materials (explant, callus/embroid and plantlets). Of these 13.4 % was *Cladosporium* sp. But, these fungal species were found to cause death of the culture material. Authors mentioned that some probable sources of contaminations such as handling of plant materials, culture vessels and the laboratory were discussed.

At *Asparagus*, co-culture phenomenon has been reported by the Cachiță and collaborators (2008), but the authors mentioned studied just only co-culture occurred *in vitro* spontaneously, not made subcultures of plant and microbial material, from a also co-culture. We realized this co-culture in order to demonstrate that, after 6 weeks, the phytoinocula does not get infected with fungus, even if it formed a fructification body, and has spores (Fig. 2). We assume that infection is inevitable, however, but in the individual subculture does not occur.

Litz and Conover (1981) mentioned that the infection would develop on the medium later. Only the latent infections may occur in the establishment stage (Reed et al., 1995).

Analyzing the fructification cone fragments of optical microscope we could highlight the mycelium of fungus and sporulated forms (Fig. 2).

A B C

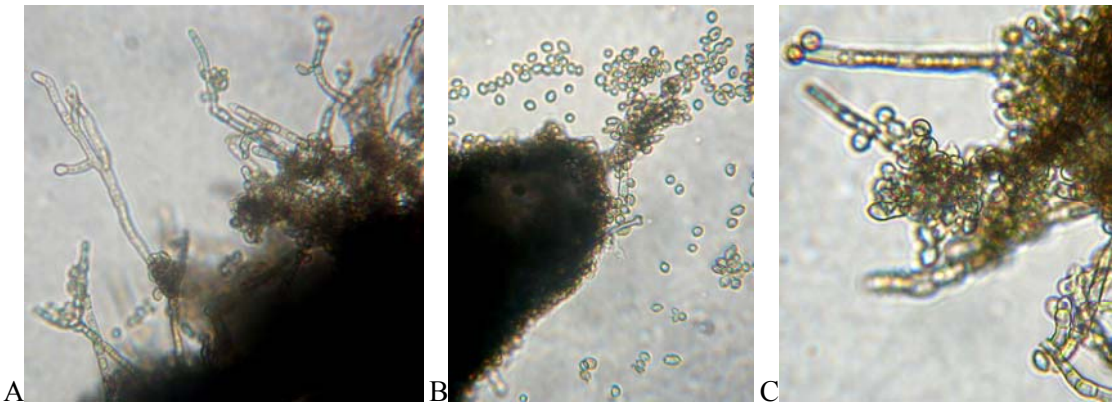

Fig. 2. *Cladosporium* sp. mycelium and spores, from fructiferous body, at 6 weeks from it spontaneous appearances on first cultures medium (A-C- 200X) (original).

From this spontaneous co-culture, we made an induced co-culture (Fig 3).

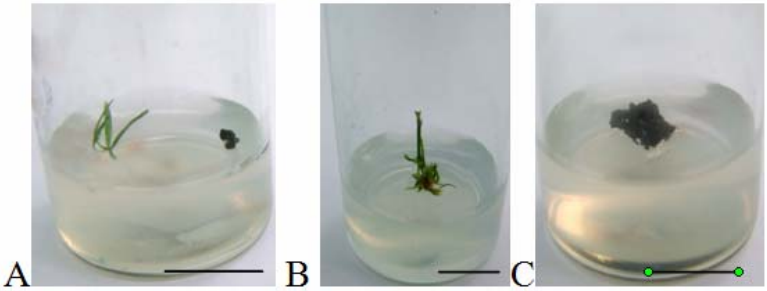

Fig. 3. Aspects of induced co-culture (A), of individual *Asparagus* phytoinocula subculture provided from spontaneous co-culture (B) and individual fungus subculture (C), in the *first day* of second induced co-culture (bars means 1 cm) (original).

At *30 days after initiation of induced co-culture*, each of the three types of culture has developed very well (Fig. 4). Thus, induced co-culture between *Asparagus* phytoinocula and fungal mycelium, both grew normally without interaction, without affecting each other (Fig. 4 A). Culture of individual *Asparagus*, provided from a spontaneous co-culture not has any form of infection, the regeneration process was complete, the vitroplantlets having an average 2.5 cm in size, which shows that although it originate from a co-culture with a fungus, phytoinocula not disease manifests, being grown as individuals (Fig. 4 B). In turn, cultivated individual fungus showed a normal growth, reaching 1 cm diameter of about a month, as well as it from co-culture, both in terms of size and shape and macroscopic aspects (Fig. 4 C).

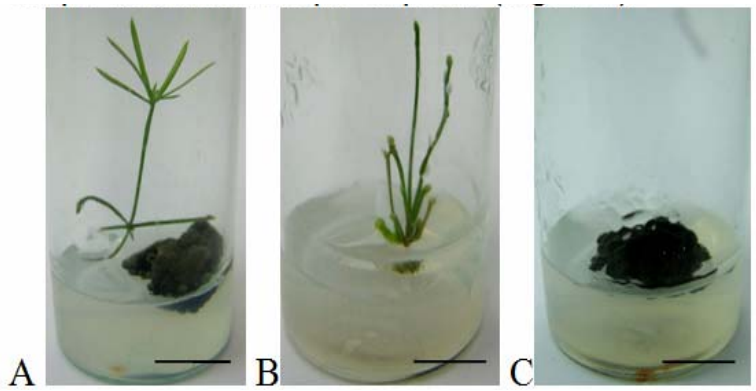

Fig. 4. Aspects of induced co-culture (A), of individual *Asparagus* phytoinocula subculture provided from spontaneous co-culture (B) and individual fungus subculture (C), at *30 day* of second induced co-culture (bars means 1 cm) (original).

In 1990, Nik and Salleh, on *Asparagus* tissue culture inoculated with *Fusarium proliferatum*, studied the surface activity to the fungus on the host and its mode of penetration. The authors used filtered suspensions and cultures of *Fusarium oxysporum*. Inoculation with conidia led to the hyphae formation in less than 24 hours, in two days there was a distinct network of hyphae on the surface tissue. At 6 days *Asparagus* stem necrosis occurred, and at 12 days it was spread throughout the stem. In some cases, at the 8th day was developed a large mycelium on the stem surface, but without the appearance of necrosis. However, in transversal section trough stem were observed red formations in subepidermal layers. *Fusarium* hyphae penetration occurred directly through the layers of epidermis rarely, because there is the cuticle layer (Nik and Salleh, 1990). In fact, cracks, stomata openings provided a quick access to underlying tissues. Infection also caused dark deposits in the vascular system to form, thus leads to wilting. Inoculation of *Asparagus* shoots with mature filtered tissue culture led to chloroses and death of infected plant in less than four days.

CONCLUSIONS

Growing *Asparagus officinalis* L. phytoinocula and a fungus in *in vitro* co-culture conditions, did not affect the two species, neither positive nor negative.

The *Asparagus officinalis* L. phytoinocula was not infected by fungus, in co-culture conditions.

For *in vitro* micropopagation the occurrence of such infections can lead to large losses for producers, but in this case, we could prove that the fungus does not affect the production of biomass, as described experiment, it can be studied in future in order to achieve symbiosis.

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