

OCCURRENCE OF ENDOPHYTIC FUNGI CAUSING RECALCITRANCE OF OLIVE CULTIVAR 'Istrska belica' DURING SHOOT CULTURE ESTABLISHMENT

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Abstract: The primary aim of this study was to establish a micropropagation procedure for the Slovenian frost-tolerant olive cultivar 'Istrska belica'. Establishing an *in vitro* culture was very difficult due to constant contaminations, tissue browning and stunted shoot growth. A sterile shoot culture was finally achieved by washing with running tap water, immersing in a mixture of ascorbic and citric acid and sterilizing with 70% ethanol and dichloroisocyanuric acid. Shoot growth was optimal on DKW medium supplemented with 4 mg/L of 2iP. Even in optimized conditions, sporadic fungal outbursts occurred. Fungi were isolated and their taxonomic origin was determined by morphological observation and molecular identification. Based on BLAST queries in the NCBI database, five genera of fungi were identified: *Cladosporium*, *Chaetomium*, *Preussia*, *Biscogniauxia* and *Sistotrema*, the last three genera being isolated from olives for the first time. A detailed literature search was performed to provide data on previous reports of these genera in relation to their putative endophytic presence and their possible pathogenic status. This is the first study reporting the presence of endophytic fungi in olive tissue culture. The information provided in this work can be very useful for the optimization of micropropagation protocols of recalcitrant olive cultivars and can potentially improve field performance of nursery plants.

Key words: *Olea europaea*; *in vitro* culture; recalcitrant micropropagation; endophytic fungal genera

INTRODUCTION

Micropropagation is a rapid propagation technique for mass production and has been used for several decades in olives (Rugini 1984). This technique allows high quality production and rapid growth of superior olive genotypes. Although several cultivars have been propagated successfully, protocols for culture establishment are still not available for several recalcitrant cultivars. This problem was emphasized by Peyvandi et al. (2009) and by Rostami and Shahsavari (2012) for *in vitro* culture of Iranian olive cultivars.

The successful establishment of shoot culture is often limited by two different factors: oxidation of explants and attempts at optimizing shoot growth conditions, and fungal contamination. In olives, the initiation stages of regeneration, where heavy oxida-

tion of explants and problems related to fungal contamination of tissues can originate in either field or greenhouse grown plants, are the most difficult.

Many sterilization procedures have been tested for different olive cultivars. Khan et al. (2002) developed a successful protocol for *in vitro* culture of cv. 'Leccino', with a sterilization procedure as follows: washing with running tap water, immersion in a solution of ascorbic and citric acid (100:150 mg/L), disinfection with 0.1% of HgCl₂, followed by immersion of explants in 50% NaOCl. Rostami and Shahsavari (2012) reported a successful sterilization procedure for cv. 'Mission', achieved by immersion of axillary buds in 50:50 citric and ascorbic acid solution, followed by immersion in 70% ethanol, then immersion in 10% sodium hypochloride. In order to control fungal outbreaks, the authors added 4 mg/L of nanosilver particles to the

initiation medium. Lambardi et al. (2013) proposed a sterilization protocol for Mediterranean olive cultivars that involved rinsing explants in running tap water and treating with a solution containing 11% NaOCl and 0.035% HgCl₂, followed by rinsing in sterile distilled water, but no data are given for the efficiency of this protocol.

Growth regulators and media manipulations are the key factors for *in vitro* proliferation and regeneration in olive. *In vitro* culture of *Olea europaea* species is widely dependent on the medium composition (Cozza et al., 1997). The first known reports on *in vitro* culture of olive are from the mid-1970s, in which researchers wanted to develop a mineral medium composition for all stages of *in vitro* culture in order to establish a micropropagation protocol suitable for all cultivars (Peixe et al., 2007). From this point of view, the most suitable media for olive micropropagation are the OM medium (Rugini 1984) and two modifications of MS medium (Murashige and Skoog 1962), the modification of MSI medium (Fiorino and Leva, 1986) and MSM medium, as modified by Leva et al. (1992). In spite of extensive studies, it was shown that all of these media compositions were not effective for all olive cultivars (Grigoriadou et al., 2002). Leva et al. (2012) noted that progress in improving the micropropagation technique has been relatively slow, due to the slow growth of olive explants and also cultivar dependency.

In addition to the mineral formulation, plant growth regulators are one of the most important components of *in vitro* culture media. Rugini (1984) reported that only one cytokinin, zeatin, induced the growth of olive explants cultured *in vitro*. Zeatin is widely used today generally at rates from 4.56 to 45.62 µM. Garcia-Feriz et al. (2002) replaced zeatin with thidiazuron and 6-benzylaminopurine (BAP) because of the high cost of zeatin. Grigoriadou et al. (2002) reported an increased number of microshoots/explants as well as increased proliferation rate but reduced shoot height when a combination of zeatin and BA was used for *in vitro* propagation of the Greek cultivar 'Chondrolia Chalkidikis'. The highest number

of new microshoots/explants was achieved when the medium was supplemented with 20 µM zeatin.

For all culture media, the energy or carbon source is another important component. Sucrose is widely used for such a purpose. In media for olive micropropagation, sucrose has often been replaced with mannitol (Leva et al., 1994).

Endophytic fungi represent a serious problem that can reduce the growth rate of seedlings and trees, and can also cause contamination of *in vitro* grown shoot cultures. Suryanarayanan (2011) reported that horizontally transmitted endophytes belong to an ecological group of fungi that infect living plant tissues and survive in them without causing any disease symptoms. In the case of olive, Sanei and Razavi (2012) reported highly infected young trees in new plantations in northern Iran. The trees wilted, and dieback and death was also recorded. The authors investigated possible causes of these phenomena and found several fungi that caused disease. *Verticillium dahliae* and *Fusicladium oleagineum* were the most common fungi on all cultivated olive cultivars but eleven other genera were also present. However, it was unclear which of them was the primary and which the secondary invader.

In our study, infections by microorganisms, especially fungi, were a serious problem in the culture. During the initiation phase of olive cv. 'Istrska belica' *in vitro*, a high number of explants lost through fungal, bacterial and browning tissues were observed.

The aim of this work was to establish an efficient *in vitro* culture protocol for cv. 'Istrska belica', a Slovenian cultivar known to be more frost-tolerant than the majority of olive cultivars. Since infections by microorganisms, predominantly fungal, were found to be a serious problem during the initiation phase, we performed isolation and determination of putatively endophytic fungal species using genetic and morphological evaluation. The possible roles of the identified fungal genera on tissue culture-grown olive shoots are discussed.

MATERIALS AND METHODS

Plant material and culture conditions

The *in vitro* culture performed in the current study was carried out on olive plant material taken from 3-year-old healthy olive trees (*Olea europaea* L. 'Istrska belica') planted in a greenhouse. Apical shoots (≈ 20 cm long) were cut into uninodal segments (about 2 cm in length) and leaves were partially eliminated. The uninodal segments were then washed under running tap water for 1 h. In order to control the production of phenolic compounds and necrosis of explants, shoot segments were immersed in a mixture of 20 mg/L ascorbic and 200 mg/L citric acid for at least 1 h. Uninodal segments were further exposed to a sterilization procedure by immersion in 70% ethanol for 1 min, washing in sterile distilled water, then immersion in 1.66% dichloroisocyanuric acid (DICA) for 10 min and washing again twice in sterile distilled water.

The nodal segments were cultured on DKW (Driver and Kuniyuki, 1984) medium, supplemented with 4 mg/L of 6-(γ,γ -dimethylallylamino)purine (2iP), 30 g/L sucrose, 7 g/L agar with the pH adjusted to 6.0, for culture initiation. The cultures were maintained in a growth chamber at $25 \pm 1^\circ\text{C}$ under cool white fluorescent lamps with a 16/8 h light/dark photoperiod.

After two months of culture, the explants developed into shoots, 3-5 cm in length. New shoots were divided into nodal segments with one pair of buds and cultured under the same environmental conditions for three months. Proliferation of explants was carried out on OM medium (Rugini 1984), supplemented with 30 g/L mannitol, 7 g/L agar, 4.8 mg/L trans-zeatin riboside and 1.49 mg/L gibberellic acid (GA_3). The pH of the proliferation medium was adjusted to 5.8. Finally, the proliferated shoots were cut into 1-cm-long leafy segments with one pair of buds, and subcultured on OM medium, supplemented with 30 g/L mannitol, 7 g/L agar, 2 mg/L trans-zeatin riboside and 1 mg/L GA_3 . The pH of the OM medium was adjusted to 5.8.

Detection of fungal contaminants

Fungal contaminants were observed during the initiation stage of *in vitro* culture of 'Istrska belica'. For fungal contaminant identification, we used sterile uninodal segments from the tissue culture, cut vertically and placed on plates with Sabouraud dextrose broth (S3306 Fluka) or yeast extract peptone dextrose (YPD) broth (Duchefa Y1708). Plates were incubated in the dark at 29°C in a growth chamber. After one month, each plate with fungi was transferred to 250-ml Erlenmeyer flasks with liquid Sabouraud dextrose broth and cultured on an orbital shaker at 120 rpm at 25°C for 10 days. DNA isolation was performed after 10 days. Small pieces of fungal mycelia were transferred from the *in vitro* culture to plates of potato dextrose agar (PDA, Biolife 4019352), oatmeal agar (OMA, Fluka 03506) and synthetic nutrient agar (SNA, Sifin TN 1017) and incubated at 25°C , 60% RH and a 12-hour dark-light (near UV) regime. Near UV light was used to induce sporulation. Subcultures on different nutrient media were examined periodically and sporulating isolates were identified based on their morphological characteristics. Identification was carried out by standard morphological techniques (fungal slide culture and microscopy).

Isolation and identification of endophytic fungi

Genomic DNA of each unknown fungal contaminant was isolated from fresh liquid cultures using a modified cetyltrimethyl ammonium bromide (CTAB) protocol for plant DNA isolation (Javornik and Kump 1993). The ITS region was amplified using primer pairs ITS1-ITS4 or ITS5-ITS4 (White et al., 1990). Amplification was performed in a 50- μl reaction volume containing PCR buffer (20 mM KCL, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 25 mM MgCl_2 , 20 mM Tris-HCl, pH 8.4), 10 mM of each deoxyribonucleotide triphosphate, 15 pmols of each primer, 10 ng template DNA and 5 units of *Taq* polymerase (Promega). The thermal cycling program was as follows: 5 min initial denaturation at 95°C , followed by 30 cycles of 30 s denaturation at 95°C , 1 min primer annealing at 52°C , 1 min elongation at 72°C , and final 5 min extension at 72°C . PCR

products were purified using the PCR Clean-Up Protocol ExoSAP-IT® (USB Europe GmbH). Purified PCR products were directly sequenced with primer pairs, as mentioned above, in an ABI 3130xl Genetic Analyzer.

ITS sequence analysis

The ITS sequences generated in this study were assembled using CodonCode Aligner (v 4.0.4) and used as a query search for similar sequences in Gene Bank with the BLASTN program to provide identification of the fungi. ITS sequences (average 658 bp) were deposited in European Molecular Biology Laboratory (EMBL) GenBank.

RESULTS

In order to establish shoot cultures of cv. 'Istrska belica', most of our *in vitro* studies were initially focused on finding a suitable sterilization procedure and culture media. These preliminary experiments included a total of 3106 nodal explants.

Sterilization treatments included running tap water treatment, brief ethanol submersion and various concentrations of DICA. Initiation media for *in vitro* establishing of cv. 'Istrska belica' were firstly based on MS medium, supplemented with various concentrations of sucrose, agar and various pH in a range from 5.6 to 6.0. Different concentrations of vitamins (pyridoxine, thiamine) and plant growth regulators such as trans-zeatin riboside, BAP, indole-3-acetic acid (IAA) and GA₃ were also tested (data not given). These preliminary experiments were not successful. In most cases, browning of tissue occurred, and fungal contaminations were also frequent, even under the most restrictive sterilization procedures.

The first successfully proliferated buds were observed a few years ago. This protocol (INI 1) included treatments with antioxidants following the sterilization treatment. Experimental design and culture response are given in Table 1. Proliferation was noted after two weeks in culture, resulting in 3.0-3.5-cm-long axillary shoots. The shoots were then subcultured

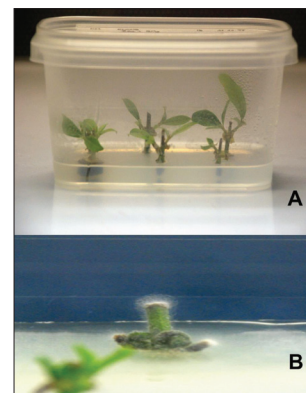


Fig. 1. Characteristic development of *in vitro* culture of 'Istrska belica'. **A** – proliferation of shoots cultured on INI1 medium; **B** – occurrence of fungal contaminants from putatively axenic explants.

on the same medium, but further development was not achieved. An improved protocol (INI 2) yielding continuous shoot culture growth (Fig. 1A) was achieved by a similar sterilization treatment as in the INI 1 experiment but with altered medium composition (Table 1). On this medium, 20 obtained shoots continued growth and could be further subcultured.

Although a small number of explants finally yielded apparently healthy and non-contaminated shoots, several other explants showed either stunted growth or the reappearance of fungal contaminants emerging from the vascular tissues of segments (Fig. 1B). In order to identify these unknown contaminants of putatively endophytic origin, indexing experiments were performed. Based on BLAST queries in NCBI, five genera of fungi were identified and were determined as *Preussia*, *Cladosporium*, *Chaetomium*, *Biscogniauxia* and *Sistotrema* (Table 2). Among the isolates, five belonged to the same genus (*Chaetomium*), while others were present as single species.

Analysis of published records on fungal genera detected in our study

In order to investigate whether the identified fungal genera should be classified as true endophytes or rather as occasional contaminants, we performed a detailed bibliographic study of references in which records of fungal status have been previously described. The em-

Table 1: Sterilization protocol and initiation media used in two experiments yielding shoot proliferation.

Number of initiation	Sterilization procedure	Medium composition	Plant growth regulators	No. of nodes from donor plant	No. of proliferated buds
INI 1	Washing with running tap running water for 1 h, immersion in 20 mg/L ascorbic and 200 mg/L citric acid for 1 h, dichloroisocyanuric acid 0.83 g/50 ml of sterile distilled water for 10 min	OM medium supplemented with 36g/L mannitol, 7 g/L agar, pH adjusted to 5.8	zeatin 4 mg/L	350	5
INI 2	Washing with running tap water for 1 h, immersion in 20 mg/L ascorbic and 200 mg/L citric acid for 1 h, immersion in 70% ethanol for 1 min, dichloroisocyanuric acid 0.83 g/50 ml of sterile distilled water for 10 min	DKW medium supplemented with 30 g/L sucrose, 7 g/L agar, pH adjusted to 6.0	2iP 4 mg/L	256	20

Table 2. Morphological and molecular identification of investigated fungal taxa based on their morphological characteristics and ITS sequences according to BLAST queries in NCBI.

Fungal isolate	Morphological identification	GenBank accession No.	Closest blast match (GenBank accession No.)	Query/reference ITS length (similarity %)
6	<i>Preussia</i> sp. (Von Arx et al., 1975; Breitenbach and Kränzlin1984)	KJ186952	<i>Preussia</i> sp JN225886.1	516/518(99%)
5	<i>Cladosporium</i> sp. (Ellis 1971; 1976)	KJ186953	<i>Cladosporium</i> sp. KF367474	529/529(100%)
7	<i>Cladosporium</i> sp. (Ellis 1971; 1976)	KJ186954	<i>Cladosporium sphaerospermum</i> KC311475	533/533(100%)
2, 3, 4, 1,	<i>Chaetomium</i> sp. (Von Arx 1981; Samuels and Blackwell 2001)	KJ186955	<i>Chaetomium</i> sp. JN209927	550/550(100%)
8	<i>Chaetomium</i> sp. (Von Arx 1981; Samuels and Blackwell 2001)	KJ186956	<i>Chaetomium globosum</i> JN209927	
9	<i>Biscogniauxia</i> sp. (Breitenbach and Kränzlin1984, Samuels and Blackwell 2001)	KJ186957	<i>Biscogniauxia nummularia</i> EF155488	593/594(99%)
10	Basidiomycota (no fruiting bodies observed, septate hyphae with clamps)	KJ186958	<i>Sistotrema brinkmannii</i> DQ899094	607/608(99%)

phasis of this analysis was the presence or absence of data considering classifications of these putative endophytes in olives or in related genera.

Cladosporium sphaerospermum Penz.

Species of *Cladosporium* are cosmopolitan and commonly encountered on all kinds of plant and debris. The most species are isolated from soil, food, paint, textiles and other organic matters or colonize as secondary invaders leaf lesions caused by plant pathogenic fungi (Ellis 1971, 1976). Other species of this genus are plant pathogenic, i.e., they are causal agents of leaf spots and other lesions (Schubert and Braun 2005). *Cladosporium* species are also known as common endophytes (Riesen and Sieber, 1985; Brown et al., 1998; El-Morsy 2000; Fisher et al., 1992).

Cladosporium sphaerospermum was first described by Penzig (1882) from decaying *Citrus* leaves and branches in Italy. This cosmopolitan species occurs as a secondary invader on numerous plants, saprobic on dead leaves, stems, wood and other plant organs, isolated from outdoor and indoor air, soil, hypersaline water, indoor wet cells, foodstuffs and other organic matter, paint, silicone, textiles and occasionally isolated from man and animals (nails, nasal mucus etc.) (Ellis 1971; Bensch et al., 2012). *C. sphaerospermum* was isolated as an endophyte from the leaves and twigs of *Ulmus macrocarpa* (Sun et al., 2012). On the basis of these records, we propose that all identified isolates of the genus *Cladosporium* could be true endophytes but with a possible role as facultative secondary invaders.

***Sistotrema brinkmannii* (Bres.) J. Erikss.**

Sistotrema brinkmannii is associated with soil, moss and wood of angiosperms and gymnosperms in natural environments and forest products. It is also considered a saprotroph producing brown rot decay (Wang and Zabel, 1990; Ginns and Lefebvre 1993). This species is geographically widespread (Ginns and Lefebvre, 1993; Breitenbach and Kränzlin, 1986) and has been isolated from a variety of substrates, including utility poles (Wang and Zabel, 1990), diseased *Pinus sylvestris* roots (Menkis et al., 2006) and decaying wood (Son et al., 2011). An *S. brinkmannii* specimen has also been isolated from *Pinus banksiana* (jack pine) and *Populus tremuloides* (quaking aspen) seedling roots, which showed no visible signs of decay, and it did not appear to affect seedling growth in pure culture synthesis. The pure culture combination of *S. brinkmannii* with sterile jack pine and aspen seedlings demonstrates an association with roots that is neither detrimental to the seedling nor typically mycorrhizal (Potvin et al., 2012). The ecological role of the association of *S. brinkmannii* with tree roots is still unclear. On the basis of these data, the presence of *Sistotrema* in olive isolates is most likely of non-endophytic origin.

***Preussia* sp. Fuckel**

Preussia and *Sporormiella* genera are very closely related, almost indistinguishable at the morphological level in pure culture, and so Guarro et al. (1997b) considered these two taxa to be congeneric according to morphological characteristics.

Preussia species have been mainly isolated from soil, wood and plant debris and also dung (Valldosera and Guarro, 1990; Guarro et al., 1997a, 1997b; Robinson et al., 1998; Chang and Wang 2009; Arenal et al., 2004, 2005, 2007). *Sporormiella* species are among the most common fungi inhabiting dung of both ruminant and non-ruminant animals (Khan and Cain 1979; Caretta et al., 1998).

Several *Preussia* and *Sporormiella* species have been reported as fungal endophytes (Fisher et al., 1992; Guarro et al., 1997a; Peláez et al., 1998; Arenal

et al., 2004, 2005; Sun et al., 2012). Arenal et al. (2007) suspected that an endophytic habit, considered relatively unusual for this genus, may actually be the rule rather than the exception, or at least as common as the coprophilous lifestyle. Fisher et al. (1992) isolated *Sporormiella intermedia* (syn. *Preussia intermedia*) from superficial disinfected twig pieces of olive (*Olea europaea*). Distinguishing the *Preussia* and *Sporormiella* genera at the morphological level is very difficult, so it is possible an unintentional mistake was made when determining the genus, because molecular identification methods were not used in this study. On the basis of the presented citations, we propose that the identified isolate of *Preussia* can be listed as a true endophyte in olives.

***Chaetomium globosum* Kunze**

Chaetomium species are distributed worldwide: in soil, on plant debris and plant materials it is known as a soft-rot fungus of softwood and hardwood timber, frequently encountered in archives, on wall paper, textiles, etc. This genus consists of about 95 widespread species (Domsh et al., 1980; Kirk et al., 2008). Several *Chaetomium* species, including *C. globosum*, have been reported as fungal endophytes of herbaceous and woody plants (Reissinger et al., 2003; Istifadah et al., 2006; Hoffman and Arnold, 2008; Oses et al., 2008; Shankar Naiket et al., 2009). On the basis of these records and frequent detection in woody plants, we propose that the identified isolate of genus *Chaetomium* could be a true endophyte.

***Biscogniauxia nummularia* (Bull.) Kuntze**

Nugent et al. (2005) found *B. nummularia* to be widely occurring as an endophyte in beech trees in southern England and parts of Wales, with the development of stromata only on freshly detached branches or on dying branches still attached to the tree. Rogers (2000) referred to such xylariaceous fungi as 'sneaky', preferring to think of them as latent invaders developing as the host becomes weakened. *Biscogniauxia nummularia* is also associated with severe beech-decline events recorded in southern Italy (Granata and Whal-

ley, 1994; Granata and Sidoti, 2004). Based on field surveys and experimental results, *Biscogniauxia nummularia* is evidently linked to canker disease in water-stressed host trees (Hendry et al., 1998, 2002; Nugent et al., 2005). On the basis of the presented citations, we propose that the identified isolate of *Biscogniauxia* can be listed as a true endophyte in olives as well as a stress, i.e., opportunistic, pathogen.

In general, all isolated fungi from the olive tissue culture have endophytic potential as well as saprotrophic, but only *Biscogniauxia nummularia* possessed some pathogenic ability (Nugent et al., 2005). *Biscogniauxia nummularia* has never previously been isolated from *Olea europaea*. The related fungus *B. nummularia* var. *rumpens* was isolated from *Fraxinus* sp., which belongs to the same family (Oleaceae) as the olive tree (Miller 1961).

DISCUSSION

This study confirms previous assumptions that clonal propagation via *in vitro* shoot culture might still be problematic for some olive cultivars. Our study identified several bottlenecks and found some solutions. We showed that relatively restrictive sterilization treatments accompanied by an antioxidant might enable the successful initiation of *in vitro* culture, while modifications in media compositions should be an additional element in enabling further shoot growth and multiplication. These findings are in agreement with previous notes, such as Lambardi et al. (2013), stating that olive micropropagation is not easy in practice due to several problems, such as oxidation of explants, difficulties in disinfection, labor intensiveness in establishing shoot cultures, and it is highly cultivar-dependent.

The term endophyte has been variously defined (Hyde and Soyong, 2008). Endophytes survive in living tissues of plants for either a short or extended period without producing any visible symptoms. Endophytes also have a cryptic existence and their main role in the ecosystem appears to be that of decomposers, since they are among the primary colonizers of

dead plant tissues (Oses et al., 2008). In spite of numerous studies, the boundaries between endophytes, weakness or stress parasites and facultative parasites are still unclear.

The main focus of this study was the identification of late contaminant outbursts, followed by the identification of fungal genera. This is the first such study in olives. Similar findings have been reported in other woody species. Omamor et al. (2007) reported 25 species of fungi belonging to 14 genera found in oil palm tissue culture. Endophytic fungi and bacteria were identified as a problem in evergreen rhododendrons cultivated *in vitro* (Purmale et al., 2012), in which the genera *Penicillium*, *Cladosporium* and *Cephalosporium* were isolated from putatively sterile shoots. Similarly, Pirttilä et al. (2003) found two fungal species isolated from pine tissue cultures originating from buds.

Although no *in vitro* determination of fungal endophytes has existed to date in olives, various studies have been published on the detection of fungal species associated with orchard-grown olive trees. In such a study, Fisher et al. (1992) identified fungi isolated from the xylem and whole stems of mature trees. The authors reported Ascomycetes, Deuteromycetes and Zygomycetes. A survey of olive fungal disease in North Iran was performed by Sanei and Razavi (2012). The authors discovered several fungal species associated with the death of young olive trees in the field or in nurseries, including some stem-decay fungi, such as species of *Ascochyta*, *Alternaria*, *Cephalosporium*, *Chaetomium*, *Cladosporium*, *Diplococcium*, *Diplodia*, *Nigrospora*, *Sphaeropsis*, *Stemphylium* and *Ulocladium*. They also warned that these fungi can be both primary and secondary invaders and proposed further work to be undertaken to confirm their importance as potential pathogens.

We can conclude that our findings are in accordance with previous reports for two of the identified genera (*Cladosporium* and *Chaetomium*), which have already been reported in orchard-grown olive trees, but three other genera, *Preussia*, *Biscogniauxia* and *Sistotrema*, have not so far been reported. Among these last three genera, our literature survey points

to *Biscogniauxia* as potentially the most problematic, since its pathogenic nature cannot be excluded.

Although our findings showed that an adequate micropropagation protocol can be established for an initially recalcitrant olive cultivar such as 'Istrska belica', special attention needs to be paid to the presence of fungal endophytic contaminants by regular indexing. We propose that further studies should be focused on the evaluation of nursery plants produced by conventional and micropropagation procedures in relation to possible differences in their growth characteristics.

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