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Tissue culture of Pyrethrum (*Tanacetum cinerariifolium*) and associated microbial contamination

Tkivna kultura bolhača (*Tanacetum cinerariifolium*) in z njo povezana okužba z mikroorganizmi

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Abstract: Microbial contamination was observed on several subcultures of Pyrethrum (*Tanacetum cinerariifolium*) (Trevir.) Schultz-bip. callus lines. The presence of microorganisms was detected by isolation of contaminants in pure culture from 7 out of 34 callus lines and direct amplification of eubacterial 16S rDNA in the pyrethrum callus and plants and isolated bacteria. Altogether 16 contaminants were further analyzed, observing their morphology on several media and restriction of amplified 16S rDNA. Analysis revealed presence and persistence of morphologically and genetically diverse bacteria in pyrethrum tissue culture. Due to cross reactivity of 16S rDNA primers with DNA of plant origin, no conclusions could be drawn on the origin of contaminants.

Keywords: tissue culture / pyrethrum / *Tanacetum cinerariifolium* / callus culture / microbial contamination / PCR

Izvleček: Po vzpostavitvi kalusnih linij bolhača (*Tanacetum cinerariifolium*) (Trevir.) Schultz-bip. smo v kulturah opazili okužbo z mikroorganizmi. Z različnimi izolacijskimi postopki in gojitvenimi metodami smo iz 7 od 34 kalusnih linij izolirali posamezne seve mikroorganizmov. Nobeden od izolatov ni bil izoliran iz več kot dveh kalusnih linij. S pomnoževanjem eubakterijske 16S rDNA in nadaljnjo restrikcijsko analizo z encimom *Mnl*I smo analizirali kalusno tkivo, rastline bolhača in izolirane bakterije. Analiza je pokazala morfološko in genetsko raznolikost bakterij prisotnih v tkivni kulturi bolhača. Zaradi navzkrižne reaktivnosti uporabljenih 16S rDNA oligonukleotidnih začetnikov, na prisotnost podobnih bakterijskih sevov v kalusnem tkivu in steblih bolhača, ni bilo mogoče sklepati na izvor okužb.

Ključne besede: tkivna kultura / bolhač / *Tanacetum cinerariifolium* / kalusna kultura / okužba z mikroorganizmi / PCR

Running title: Tissue culture of Pyrethrum and associated microbial contamination

Introduction

Pyrethrum *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip. Asteraceae is a source of the natural, non-persistent insecticides pyretrine, an environmentally friendly and efficient method of insect control (Jovetić and de Gooijer 1995), which is therefore interesting for *in vitro* biotechnological production (Hitmi et al. 2000). The establishment of pyrethrum tissue culture was successful from shoot cultures and initial explants from leaves and flowers (Gaspan et al. 2004).

The management of microbial contamination of plant cell and tissue cultures is a critical point

in commercial and research tissue culture laboratories. Epiphytic and endophytic microorganisms, introduced with the explants or poor aseptic technique, are major causes of contamination of tissue culture. Of special importance are contaminants that are commonly not visible but influence tissue culture quality (Leifert and Cassells 2001, Herman 2004, Herman 2007).

We have reported successful establishment of pyrethrum tissue culture however, despite rigorous sterilization of plant tissues prior to establishment of tissue cultures, contaminations were observed in callus cultures after several subcultures (Bergant et al., 2005). Contaminants were sensitive to antibiotic treatment (Bergant et al. 2005), indicating their microbial origin. Since identification of contaminants is a key step in their effective control we have attempted their isolation in pure culture. Their origin was researched through amplification of 16S rDNA from callus cultures and isolated bacteria after *Mnl*I restriction analysis of PCR products.

Material and methods

plant material: Tissue culture was initiated from flower heads and shoots of Pyrethrum *Tanacetum cinerariifolium*. The flower heads and shoots were collected in the Botanical Garden of Ljubljana, Slovenia, and in the plant's native region on the island of Cres, Croatia (Ambrožič-Dolinšek et al. 2007). Callus and shoot cultures were maintained on Murashige and Skoog (MS) medium as previously described (Gaspan et al. 2004; Bergant et al. 2005, Ambrožič-Dolinšek et al. 2008). Cultures were kept at 23±2°C, with a photoperiod of 16 h at 50-80 $\mu molm^{-2}s^{-1}$ (Osram L 58W/77 – Fluora).

Isolation and characterization of contaminants: Upon establishment of pyrethrum callus cultures, visible contaminations were observed as different types of clouding and veils after several subcultures (Fig. 1).

The morphology of these contaminations was described (Tab. 1). The calli and surrounding contaminated media were inoculated into media supporting bacterial and fungal growth (Tab. 1) in an attempt to isolate them in pure culture: liquid and solid BHI (brain heart infusion agar), YMGA (yeast extract maltose glucose agar), M102 (yeast extract glucose agar with bacto pepton), YGA (yeast extract glucose agar), CMM (cornmeal – malt extract agar). Colonies were subcultured, purified and stored in glycerol at –80 ºC. Visible contamination was sporadically also observed in shoot culture, associated contaminants were isolated as described above and included in analyses (Tab. 1).

DNA isolation and restriction analysis: DNA was isolated from the plant material and pure cultures according to procedures described by Webster and Barker (1994) and Wozniak (1997), respectively. The 16S rDNA genes were amplified with the eubacterial primers 27f and 1495r (Bianciotto et al. 1996). The amplified products were cut with *Mnl*I and *Rsa*I restriction nucleases. To check for the origin of contamination, the restriction profiles of amplified products from plants and tissue culture were compared with the restriction profiles obtained from isolated pure cultures (Fig. 2). DNA isolated from other plants (greenhouse-grown pyrethrum, plum leaves) and bacteria were used as controls (data not shown).

In-silico analysis: The expected fragment lengths of PCR products generated by eubacterial primers 27f and 1495r (Bianciotto et al. 1996) after restriction with *Mnl*I and *Rsa*I were predicted using a combination of sequence data from NCBI (http://www.ncbi.nlm.nih.gov/BLAST) analyzed by BLAST 2.0 and REBASE (Altshul et al., 1990, 1997, Roberts et al. 2007). Predicted fragments were compared to fragments obtained by digestion of 16S rDNA amplified from isolated DNA.

Results and discussion

Callus culture of pyrethrum was successfully initiated however; microbial contaminations were observed in all established callus lines after several subcultures (34 lines, Fig. 1, Tab. 1). Similar, but less evident, contaminations could occasionally be observed also in pyrethrum shoot cultures. Persistent contamination of tissue culture was reported previously in another member of the *Tanacetum* genus: tansy (*T. vulgaris*; Keskitalo et al. 1998b). The less pronounced contaminations in shoot cultures may be connected to plant defence responses in organized shoot tissues, since

it is known that plant defence responses limit endophytic bacteria populations inside plants (Rosenblueth and Martinez-Romero, 2006).

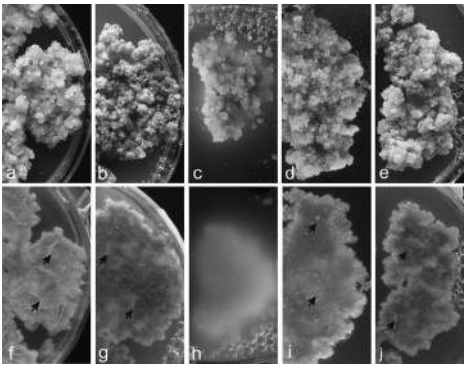

Fig. 1: Different callus lines (a, f – L52a , b, g – L16, c, h – L7 , d, i – L4, e, j – L2) 3 weeks after subculture on medium; upper callus culture sides (a, b, c, d, e) and lower callus culture sides (f, g, h, i, j); arrows indicate clearly visible contamination. Bar: 2 mm.

Slika 1: Različne kalusne linije (a, f – L52a , b, g – L16 , c, h – L7 , d, i – L4, e, j – L2) tri tedne po prestavljanju na gojišče; zgornja stran kalusa (a-e) in spodnja stran kalusa (f-j); puščice označujejo jasno vidno okužbo. Merilo: 2 mm.

The contaminations of callus cultures became visible after stabilization of callus growth as turbidity between and inside callus clumps and as white clouding or veils in the immediate surrounding media. Despite visible contamination, the calli were not affected in growth and morphology. However, attempts to characterize these contaminations were undertaken. Previous results based on antibiotic treatment of calli (Bergant et al., 2005) indicated that the contaminations are microbial in nature. Bacterial contaminants can remain undetected over several subcultures, since media for plant tissue culture are not optimal for their growth. In neither callus nor shoot cultures was contamination observed away from plant tissue which could indicate that the plant material itself is the source of contaminations.

The isolation of microorganisms was attempted by different isolation and culturing methods for the calli, the shoots and the immediate surrounding media. We tested 16 different isolation procedures and 14 different growth media (data not shown) however, only a few led to successful isolation of microorganisms in pure culture (Tab. 1). Altogether 16 isolates were isolated and cultured from 7 out of 34 callus lines and 7 lines from shoots. Isolates included both slow- and fast-growing strains of diverse morphology, varying from white and smooth, gray and smooth or orange and smooth to white and wrinkled. Morphologically similar strains were sometimes isolated from the calli, the media surrounding the calli and from the shoot: i.e. white and smooth, slow-growing strains were isolated from one callus line and the media around the basal part of the shoots. However, none of the strains was isolated from more than two callus lines. The varied morphology of isolates corresponds to the different morphology of contamination in association with plant tissues.

Since it is known that a range of bacteria is not accessible to cultivation method, because of their unknown growth requirements or their entrance into a viable but not culturable state (Sessitsch et al. 2002) an improvement of cultivation methods was attempted included tissue indexing and supplementing media with plant extracts, but these also failed to identify a common contaminant in different lines (data not shown).

Further, we attempted to assess the contaminations and relations among them using direct amplification of the eubacterial 16S rDNA gene and restriction analysis. Employing primers 27f and 1495r (Bianciotto et al. 1996) for amplification of 16S rDNA PCR products were obtained from isolates, calli, shoot cultures and pyrethrum plants and analyzed by restriction with *Mnl*I (data not shown).

Table 1: The isolation procedure and growth media used for microorganisms.
Tabela 1: Izolacijski postopki in uporabljena gojišča za mikroorganizme

Source of micro-organisms	**Isolated strains - morphological characteristics and type of growth**	**Isolation procedures and media**	**Media used for growing microorganisms**
Callus lines L4, L42, **L9**, **L83**, **L2**, L5	**L2/B20** (white and wrinkle, fast-growing), **L9/B19** (white and smooth, slow-growing), **L83/B18a** (gray and wrikle, fast-growing), **L83/B18b** (gray and wrikle, fast-growing), **L83/B18c** (gray and wrikle, fast-growing),	Infected calli in PBS buffer. Vortexing. Supernatant was spread to BHI and MS (1:1).	YMGA, BHI,
Callus lines L4	L4/B33 (gray and smooth, fast-growing)	Infected suspension culture from callus L4. Supernatant was spread to BHI and MS (4:1) or YMGA and MS (4:1).	YMGA, BHI
Callus lines L2, **L4**, **L30b**, L37	L30b/B32 (orange wrinkle, fast-growing)	Infected calli in MS medium. Supernatant with cells or without them was spread to YMGA.	YMGA,
Callus lines L4, **L39**, L37, L31a, L55j, **L7**	**L7/B38** (white and smooth, slow-growing) L39/B36 (gray and smooth, fast-growing)	Tissue maceration inM102 or MS. Supernatant was spread to different media (M 102, BHI, YMGA).	M102, YGA, CMM
Pyrethrum shoots	**B40** (white and smooth, slow-growing) B41 (white and smooth, slow-growing), B42 (white and smooth, slow-growing) B43 (white and smooth, slow-growing) **B44** (white and smooth, slow-growing) **B45** (white and smooth, slow-growing) **B46** (white and smooth, slow-growing)	White clouds in growth media around the basal part of the pyrethrum shoot were spread to different media (BHI, YMGA).	BHI, YMGA

In analysis of isolates PCR products were generated from all analyzed contaminating strains. Restriction analysis confirmed differences between morphologically different strains (Fig. 2). In addition, some morphologically similar strains had different restriction profiles, owing to the higher resolution of this method compared to morphological characterization. Strains L83/B18a and L30b/B32 isolated from two different callus lines exhibited the same morphology and *Mnl*I restriction pattern.

Profiles obtained from plant samples were identical for all callus lines and even pyrethrum stems, while no identity was observed between profiles of plant samples and bacterial isolates. This indicated that either (i) original microflora of plants in all these instances is identical or (ii) restriction profiles did not reflect diversity observed in morphology of visible contamination and isolated bacteria. In further testing, a profile identical to the ones observed in pyrethrum was generated from plum leaves (data not shown),

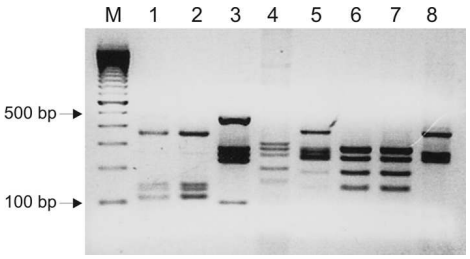

Fig. 2: Restriction fragment analysis with *Mnl*I enzyme of callus lines and isolated bacterial strains. M, marker (100 bp, Gibco). 1 – callus line L2, 2 – callus line L4, 3-8 – isolated bacterial strains M1-1, L4/B33, L83/B18a, L83/18b, L83/B18c, L30b/B32.

Slika 2: Analiza restrikcijskih fragmentov kalusnih linij izoliranih bakterijskih sevov, rezanih z *Mnl*I encimom. M, označevalec (100 bp, Gibco). 1 – kalusna linija L2, 2 – kalusna linija L4, 3-8 – izolirani bakterijski sevi: M1-1, L4/B33, L83/B18a, L83/18b, L83/B18c, L30b/B32

supporting the hypothesis that microflora, that is expected to be different between such diverse plants and environments, is indeed not represented in the profiles.

The potential of cross-reactions of 16S rDNA primers with plant DNA, presumably chloroplasts, has been recognized previously (Dent et al. 2004; Cankar et al. 2005). We have confirmed this potential through in-silico analysis of available nucleotide sequences of examined plants in NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST). In the absence of *Tanacetum cinerariifolium* target sequence, analysis was done on chloroplasts of other flowering plants including the Asteracea family. Based on in-silico annealing of primers and restriction analysis with *Mnl*I using REBASE (Roberts et al. 2007), different profiles were predicted for chloroplast DNA of different plant species with fragments of 350, 144, 132, 119, 108, 106, 94, 84 and a number of smaller fragments predicted in the Asteraceae family. However, many predicted bands are below 100 bp and would not be visible on agarose gels under our experimental conditions and the bigger predicted bands were identical to the ones observed in our experiments (350 bp, range of 106 to 144 bp). Considering the excessive amounts of chloroplast DNA in comparison to the DNA of microflora, it is not surprising that amplification of total DNA from plant tissues showed no differences among our samples. This effectively prevents us from drawing conclusions about the source of contaminants in pyrethrum cultures.

The primers used in our study have been previously used for detection of endophytic bacteria in plant tissue (Cankar et al., 2005; Thomas et al., 2007). In light of potential cross reactions of 16S rDNA primers with at least some chloroplast DNA, caution is needed in interpretation of results obtained on material that contains DNA of plant origin.

The cross-reactivity of 16S rDNA primer with plant DNA prevented us from drawing any conclusions on the origin of contamination in the tissue cultures of pyrethrum. To address this issue a different set of primers would be needed. However, results have shown that morphologically and genetically diverse bacteria can be associated with and persist in pyrethrum tissue cultures.

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