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Chemical and genetic characterization of calli derived from somatic hybridization between tansy (*Tanacetum vulgare* L.) and pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.)

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Abstract Pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.) produces environmentally benign pesticides, the pyrethrins, and tansy (*Tanacetum vulgare* L.) lower terpenes of variable biological effectiveness. As an approach to improve the oil content and composition of tansy for enhanced biological activity, a somatic hybridization technique between tansy and pyrethrum was established. About 1×10^6 of leaf-mesophyll protoplasts of both species were mixed and fused with a solution containing 15% polyethylene glycol. Light-green and yellowish calli developed from the fusion experiments. The fusion-derived calli grew vigorously on MS medium supplemented with 6.4 mg l^{-1} of BAP, 0.8 mg l^{-1} of NAA, and 30–40 g l^{-1} of glucose. Nuclear DNA content, RAPD patterns, and volatile compounds were analyzed to determine the hybridity of the calli. The nuclear DNA content of the tansy and pyrethrum genotypes, and the protoplast-derived calli of tansy were 6.41, 7.39, 13.84, and 8.11 pg, respectively. The nuclear DNA content of individual calli derived from the protoplast fusion between tansy + tansy ranged from 8.84 (F43A) to 19.59 pg (F43C) while those of the tansy + pyrethrum fusions were 10.66 (F46A) and 31.87 pg (F46B). Using four 10-mer primers a total of 56 RAPD-PCR fragments were produced. The distance matrices of fragments were calculated by average linkage cluster analysis. Two visually separated clusters were observed. One cluster consisted of the two tansy genotypes and the fusion-derived callus F43A; the other consisted of pyrethrum and fusion-derived calli F46B and F46C. Volatile compounds, such as decadienal, artedouglasia oxide, heptadecane, syringaldehyde and coniferyl alcohol, analyzed by gas chromatography mass spectrometry, were found only in the protoplast fusion-derived calli F43A and F46B. Several less volatile compounds were also detected only in fusion calli. Hexadecanoic and linoleic acids were common to fusion-derived calli and tansy, and one unknown compound to fusion-derived calli and pyrethrum. Pyrethrins I and II were detected from pyrethrum, but not from the fusion-derived calli. The additive nuclear DNA content of protoplast fusion-derived calli and the results of the RAPDs suggest that interspecific fusions had occurred. The small number of volatile compounds detected from both the fusion calli and from the donor species indicates that the unorganized callus tissue is unable to produce tissue-specific volatile compounds.

Key words *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip. · *Tanacetum vulgare* L. · Protoplasts · Somatic hybridization · Flow cytometry · Gas chromatography/mass spectrometry (GC-MS) · Random amplified polymorphic DNA (RAPD)

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Introduction

Tansy (*Tanacetum vulgare* L.) and pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.) are perennial and herbaceous species, both of which produce

bioactive terpenes. Tansy is adapted to the cold climate of the Northern hemisphere where it is widely spread (Virrankoski and Sorsa 1968; Heywood 1976; Jalas 1991). Large flower heads of tansy (Keskitalo et al. 1998) contain essential oils which are antifungal (Héthelyi et al. 1991), antibactericidal (Stefanovic et al. 1988; Holopainen and Kauppinen 1989; Héthelyi et al. 1991), insect repelling (Schearer 1984; Suomi et al. 1986; Nottingham et al. 1991; Hough-Golstein and Hahn 1992), or anti-inflammatory (Mordujovich-Buschiazzo 1996). The bioactivity of tansy has been utilized in the past in the production of fragrances, cosmetics, balsams, spices, preservatives and homeopathic medicines, and as insect repellents (Chandler et al. 1982; Mitich 1992). Pyrethrins produced by pyrethrum are a mixture of six esters derived from chrysanthemic and pyrethric acids (Head 1969), and are used as pest control agents in agriculture, horticulture (Silcox and Roth 1995), indoors (Kennedy and Hamilton 1995), as well as in veterinary use for animals (Gerberg 1995).

The interest in sustainable agriculture has increased the demand for plant-derived pesticides which can be less toxic both to mammals (Schoenig 1995) and to the environment (Gabriel and Mark 1995) than the more-stable synthetic agrochemicals. The applicability of tansy oil as a pesticide is limited because of the variable effectiveness of the different terpenes in this oil (Schearer 1984; Stefanovic et al. 1988; Holopainen and Kauppinen 1989; Héthelyi et al. 1991) and because of the low concentration of the oil in plant tissue (Dobos et al. 1992). Therefore, interspecific protoplast fusion has been one approach for improving both oil content and composition. Somatic hybrids between *Nicotiana tabacum* and *Nicotiana debneyi* contained non-volatile organic and fatty acids, phenolics, pigments and alkaloids, the concentration of which were less than, similar to, in excess of, or intermediate to those in the parental species (Court et al. 1992). Similarly, an intermediate chemotype of a somatic hybrid between peppermint (*Mentha piperita*) and gingermint (*Mentha gentilis*) (Sato et al. 1996) or spearmint (*Mentha spicata*) (Krasnyanski et al. 1998) contained terpenes inherited from both of the donor species.

There are about 1300 genera in the family Asteraceae (Heywood and Humphries 1977). In general, this plant family is quite recalcitrant in tissue culture. However, somatic hybrids between sunflower and *Helianthus gigantheus* L. (Krasnyanski and Menczel 1995); *Lactuca sativa* and *Lactuca virosa* (Matsumoto 1991); *Rudbeckia hirta* and *Rudbeckia laciniata* (Al-Atabee et al. 1990); and between *Senecio fuchsii* Gmel. and *Senecio jacobaea* L. (Wang and Binding 1993) have so far been recovered in this plant family.

We are interested in combining the insecticidal properties of tansy and pyrethrum and to this end have developed tissue culture and protoplast techniques (Keskitalo and Pehu, unpublished; Keskitalo et al. 1995) for tansy. Previously, tissue culture has been applied to tansy only to study terpene accumulation in the undifferentiated callus (Banthorpe et al. 1986; Banthorpe and Brown 1989; Svoboda et al. 1995). Micropropagation (Wambugu and Rangan 1981; Karki and Rajbhandary 1984), explant regeneration (Paul et al. 1988), embryogenesis (Pal 1992) and the production of pyrethrins in callus cultures (Kuech et al. 1985; Zito and Tio 1990; Rajasekaran et al. 1991; Sarker and Pal 1991; Dhar and Pal 1993) have all been developed for pyrethrum. Protoplasts isolated from pyrethrum resulted in callus formation (Malaure et al. 1989) but no shoots were regenerated. From isolated mesophyll protoplasts of tansy we obtained root formation (Keskitalo et al. 1995) and sporadic shoot growth (Keskitalo and Pehu, unpublished). These results encouraged us to pursue the fusion of tansy and pyrethrum mesophyll protoplasts.

The objectives of the present study were: (1) to develop a somatic hybridization method for leaf mesophyll protoplasts of tansy and pyrethrum, and (2) to characterize the nuclear DNA content, RAPD-PCR profiles and GC-MS chromatograms of the protoplast-fusion-derived calli of tansy and pyrethrum.

Materials and methods

Plant materials

In vitro grown tansy genotypes (*Tv* 142094 and *Tv* 50) and the pyrethrum clone (*Tc* 22) were cultured on MS-medium (Murashige and Skoog 1962) without hormones in a growth chamber at $24 \pm 2/20 \pm 2^{\circ}C$ (day/night) under a 16-h photoperiod with 40 μmol m^{-2} s^{-1} from fluorescent lamps. Leaf tissue was used for protoplast isolation, flow cytometry, and GC-MS analysis. In addition, two protoplast-derived calli lines, *Tv* 142094 derived from tansy genotype *Tv* 142094 and *Tv* 93 produced from our previous study (Keskitalo et al. 1995), were included in the flow cytometry analysis, together with the micropropagated pyrethrum clones *Tc* 18, 21, and 24. These clones were maintained in tissue culture and originated from shoot tips of greenhouse-grown pyrethrum plants. For GC-MS analysis pyrethrum (*Tc* 22) was cultured in the greenhouse in 3-l pots filled with peat and verimiculite (3 : 1), watered, and fertilized with a 0.1% N-P-K solution once a week.

Isolation of protoplasts

Protoplast isolation from leaf tissue of *Tv* 142094, *Tv* 50, and *Tc* 22 was carried out as described previously for tansy and pyrethrum (Keskitalo et al. 1995). The composition and concentration of the macerating enzymes were 0.125% (w/v) Macerozyme R-10 (Yakult, Honsha), 0.25% (w/v) Cellulase R-10 (Onozuka), 0.25% (w/v) Cellulysin (Calbiochem), 0.125% (w/v) Driselase, and 0.02 (w/v) Pectolyase Y-23 in 0.5 M sucrose and 5.0 mM of $CaCl_2 \cdot 2H_20$ (pH 5.6–5.7).

Protoplast fusion

Washed protoplasts of tansy genotypes *Tv* 142094 and *Tv* 50 were mixed equally (1 : 1) and re-suspended with W5 (Menczel et al. 1981)

to a concentration of 2×10^6 protoplasts ml^{-1}. Leaf protoplasts of pyrethrum *Tc* 22 were adjusted to the same concentration. In the first fusion (F43) two equally mixed tansy genotypes were employed. In the second fusion experiment (F46) an equal mix of protoplasts of the tansy genotypes *Tv* 142094 and *Tv* 50 were mixed in a 1 : 1 ratio with pyrethrum *Tc* 22 and fused. The fusion procedure was performed as described by Thomzik and Hain (1988). The fusion solution contained 15% polyethylene glycol (PEG; MWt 8000), 60 mM $CaCl_2 \cdot 2H_2O$, 90 mM mannitol, 25 mM glycine, and 10% DMSO. Eight drops of the fusion solution were pipetted by a Pasteur pipette in two spots on a Petri dish (diameter 10 cm). Three to four drops of the mixed protoplast solution were pipetted between these spots. One drop of the fusion solution was added to each drop of the coalesced fusion solution – protoplast mixture, and incubated for 10 min. Aliquots of 0.4, 0.8, 2.0, 4.8, and 12.0 ml of W5 solution containing 50 mM MES were added once to the Petri dish every 5 min. Protoplasts were incubated for 1.5 h in the dark at room temperature, collected in a glass tube and centrifuged at $50 \times g$ for 10 min. Protoplast density was determined with a haemocytometer and the viability of cells tested using fluorescein diacetate (FDA; 0.5% in acetone).

Protoplast culture

Fused and non-fused (control) protoplasts were counted and resuspended at a density of 3×10^5 protoplasts ml^{-1} in modified MS medium (Keskitalo et al. 1995) with the following specifications. The culture medium was supplemented with 5 mg l^{-1} of NAA and 1 or 5 mg l^{-1} of Zeatin. The protoplasts were cultured in 5-cm-diameter Petri dishes in the dark at $29 \pm 1°C$ until colonies were visible. Calli of 2 mm in diameter were transferred to MS medium supplemented with 0.8, 1.6 or 3.2 mg l^{-1} of NAA, 1.6, 3.2, or 6.4 mg l^{-1} of BAP, 30 or 40 g l^{-1} of sucrose or glucose, and 6.3 g l^{-1} of agar (Sigma) (pH 5.8). Calli were transferred to fresh medium every 20–30 days and cultured at $24 \pm 2/20 \pm 2°C$ (day/night) under a 16-h photoperiod at 40 μmol m^{-2} s^{-1} from fluorescent lamps.

Determination of nuclear DNA content

Nuclear DNA content (2C value) was determined by flow cytometry from fusion-derived calli (F43A, B, C, and F46A, B), protoplast-derived calli of tansy (*Tv* 142094; *Tv* 93 Keskitalo et al. 1995), leaf tissue of cultured tansy (*Tv* 142094 and *Tv* 50), and pyrethrum (*Tc* 18, *Tc* 21, *Tc* 22, and *Tc* 24). The solutions and sample preparations were prepared as described earlier (Arumuganathan and Earle 1991; Valkonen 1994; Keskitalo et al. 1998). Barley nuclei (cv Sultan, 2C = 10.94 pg; Valkonen 1994) were added to the preparation of tansy nuclei as an internal standard. The relative fluorescence of the propidium iodide-stained nuclei (1750 ± 1250 nuclei per measurement) was measured with a Coulter Epics Elite flow cytometer (Hialeah, Florida) and a 488-nm argon laser, at the Cytometry Laboratories, Department of Basic Medical Sciences, Purdue University, or as described earlier (Valkonen 1994; Keskitalo et al. 1998). The DNA content in the nuclei of the target tissue was determined relative to the 2C-DNA values of barley. Flow cytometry analysis was repeated at least two times per sample, except for callus F43B which was analyzed only once due to the lack of callus tissue.

DNA extraction, isolation and polymerase chain reaction (PCR)

Total DNA was extracted from fusion-derived calli F43A, F46B and F46C, from leaves of the tansy genotypes *Tv* 142094 and *Tv* 50, and the pyrethrum line *Tc* 22. Plant material (500 mg) was washed with distilled water and frozen in liquid nitrogen. The small-scale DNA extraction procedure was carried out as described by Hillis and Moritz (1990), and Colosi and Schaal (1993), and the DNA concentration determined as described by Keskitalo et al. (1998). RAPD-PCR reactions were performed from the above-listed six samples of DNA and from two artificially mixed DNA samples. Total cellular DNA from *Tc* 22 was mixed 1 : 1 with total DNA from *Tv* 142094 or *Tv* 50. The sample preparation for RAPD-PCR, the RAPD-PCR reaction, and the run of samples were all carried out as described by Keskitalo et al. (1998). Four random 10-mer primers (OPA 5, 11, 12, and 19; Operon, Alameda Calif.) were used for the RAPD-PCR. Analysis with each primer was carried out at least three times.

Solvent extraction

Leaves of greenhouse-grown pyrethrum *Tc* 22 (50 g), leaves of tissue-cultured *Tv* 142094 (0.5 g), the protoplast-derived callus of *Tv* 142094 (2 g), and two fusion-derived callus clones, F43A (2 g) and F46B (2 g), were extracted with dichloromethane. Air-dried plant materials were weighed, ground in a mortar, and the suspension poured into glass tubes (20 ml) filled with 10 ml of dichloromethane. Tubes were closed with a rubber cap wrapped with foil, shaken vigorously, and left to stand for 2 h. The clear solvent layer was filtered into a conical flask through a Pasteur pipette filled with cotton. The extraction was carried out twice. The extract was dried with anhydrous sodium sulphate for 3–4 days in the dark at 4°C, and evaporated to a volume of 0.5 ml.

Gas chromatography/mass spectrometry analyses

The 70-eV electron-impact mass spectra were obtained on a Hewlett-Packard (Palo Alto, Calif.), model 5890, gas chromatograph linked to a Hewlett-Packard, model 5890, mass selective detector, with a J & W Scientific (Rancho Cordova, Calif.) DB5 fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was programmed as follows: from 80°C (isothermal for 2 min) to 260°C at 3°C/min and an isothermal period of 10 min at 260°C, for a total run time of 72 min. The linear velocity of the carrier gas (helium) was 35 cm/s at 100°C. Samples of 1.0 μl were injected with a split ratio of 1 : 10. Qualitative analysis was based on comparison with a mass-spectral library (NBS75K).

Statistical analysis

Statistical analysis of the RAPD patterns was carried out as described earlier (Keskitalo et al. 1998), where reproducible PCR-bands were scored on the basis of the presence (1) or absence (0) of a fragment. The SAS program (SAS Institute 1984) modified by Levy et al. (1991) was used to calculate the similarity and distance matrices from RAPD-PCR profiles and to create the dendrogram from distance matrices. The similarity and distance matrices were calculated according to Nei and Li (1979), and the dendrogram was synthesized by an average linkage-cluster analysis.

Results

Protoplast fusion

In total 48 fusion experiments between tansy and pyrethrum protoplasts were carried out, of which three resulted in callus formation. The volume of the fusion solution was found critical for the fusion procedure. The optimum volume of the PEG solution was

1.5 × the volume of the protoplasts. If the volume was less (1 ×) no fusion occurred, and if it was higher (2 ×) the protoplasts collapsed. Protoplast viability before and after the fusion was 90–95% and 30–50% respectively.

Culture of protoplasts and fusion-derived callus

Fused tansy and pyrethrum protoplasts [F46; (*Tv* 50 + *Tv* 142094) + *Tc* 22] began to divide in 2 days, whereas the protoplasts of the intra-specific tansy fusion (F43; *Tv* 50 + *Tv* 142094) started to divide 5 days after protoplast fusion. Two weeks after the fusion small microcolonies containing 30–50 cells were observed in F46, whereas the cells of F43 had reached only the 5-cell stage. Calli formed from fusion F46 were 1-mm diameter in 20 days after fusion compared to F43 which needed twice that time to reach the same diameter. No divisions were observed in the control plates of tansy and pyrethrum protoplasts.

In total, five calli were obtained from the F43 fusion and 12 from the F46 fusion. The calli derived from F43 grew slowly and were friable, unorganized, albino or yellowish, and colored with anthocyanin. In 6–10 months the first signs of organization, such as the presence of globular structures, were observed. Callus growth was better, or similar, in MS medium supplemented with 30 or 40 g l^{-1} of glucose instead of sucrose. Calli derived from the F46 fusion grew fast and were compact, green, and formed globular and meristematic structures. Clear differences in the growth rate and structure between the callus clones derived from the fusion were observed during the 20 months of culture.

Nuclear DNA content of the parental species and fusion calli

The nuclear DNA contents of *Tv* 142094, *Tv* 50, and protoplast-derived calli of *Tv* 142094 and pyrethrum were 6.41, 7.39, 8.09–8.15, and 13.16–14.76 pg, respectively whereas the DNA content of five fusion-derived calli (F43A, B, C and F46A, B) ranged from 8.84 to 31.87 pg (Table 1).

RAPD patterns of the fusion calli

The DNA yields from fresh calli and leaves were 0.7–0.8 μg and 1.2–1.6 μg of total DNA per 100 mg, respectively. Four RAPD primers were employed, which produced a total of 56 reproducible bands

Table 1 Nuclear DNA content (pg) of tissue-cultured tansy and pyrethrum; protoplast-derived calli and protoplast fusion-derived calli. Barley (cv Sultan, 2C = 10.94 pg; Valkonen 1994) was used as an internal standard

Source	2C				4C			
	n[a]	*DNA* pg	*SD*	*Average* nuclei/run	*n*	DNA pg	SD	Average nuclei/run
Tissue-cultured pyrethrum								
Tc 22	6	13.60	0.546	1365				
Tc 18[b]	3	13.16	0.870	92				
Tc 21[b]	4	14.43	0.569	90				
Tc 22[b]	5	13.37	0.134	500				
Tc 24[b]	5	14.76	1.220	531				
Tissue-cultured tansy								
Tv 142094	5	6.41	0.115	2437				
Tv 50	5	7.39	0.301	2390	3	13.71	0.700	313
Protoplast-derived calli of tansy								
Tv 142094	2	8.15	0.227	608				
Tv 93[b]	3	8.09	0.581	748	3	16.18	1.078	90
Calli derived from intraspecific fusion F43 (*Tv* 142094 + *Tv* 50)								
Callus F43A	3	8.84	0.552	831				
Callus F43B	1	15.96	–	623				
Callus F43C	4	19.59	0.532	1610				
Calli derived from interspecific fusion F46 [(*Tv* 142094 + *Tv* 50) + *Tc* 22)]								
Callus F46A	3	10.66	1.919	522				
Callus F46B	5	31.87	1.555	2127				

[a] The number of runs carried out from the explant
[b] Flow cytometry conditions as described by Keskitalo et al. (1998)

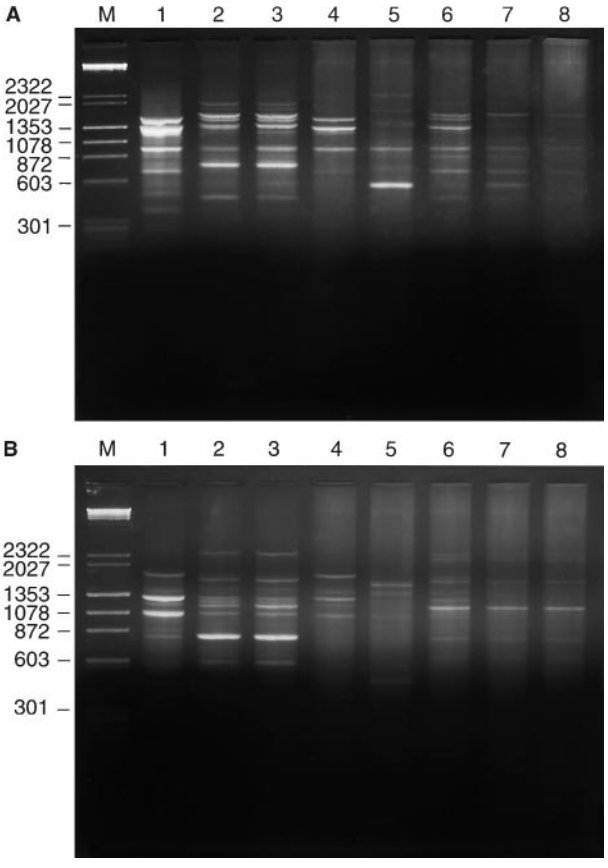

Fig. 1 RAPD profiles of samples using the random 10-base oligonucleotide primers OPA-11 (**A**) and OPA-19 (**B**) for amplification. *Lanes* (*1–8*) correspond from left to right: protoplast-fusion derived calli (F43A, F46B, F46C), parental species of tansy (*Tv* 50, *Tv* 142094), two artificially mixed samples of tansy and pyrethrum (*Tv* 50 + *Tc* 22, *Tv* 142094 + *Tc* 22), and pyrethrum (*Tc* 22). *Lane M* is the molecular-weight standard which is a mixture of (1.3 : 1 w/w) of *Hin*dIII-digested λ-DNA (Promega) and *Hae*III-digested Φ*x* 174 DNA (promega), for which the sizes are indicated

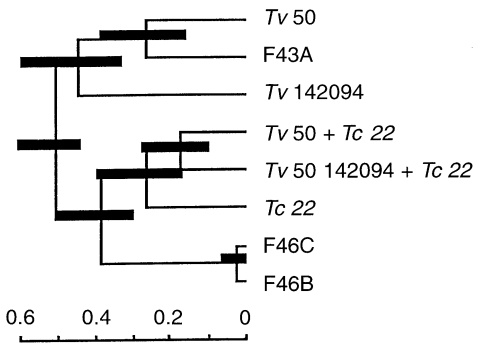

Fig. 2 Phylogenetic analysis of two tansy genotypes, pyrethrum, protoplast-fusion-derived calli and two artificially mixed samples of tansy and pyrethrum DNA based on RAPD analysis. The genetic distance between species was calculated as described by Nei and Li (1979) using the average linkage cluster analysis. The scale below the dendrogram illustrates the average genetic distance between groups

(350–2350 bp) (Fig. 1) of which four were common to all samples and 52 were polymorphic. The dissimilarity was the widest (0.714 and 0.762) between pyrethrum and the two tansy genotypes (*Tv* 142094 and *Tv* 50), and the smallest (0.031) between the two callus clones derived from the fusion F46 (F46B vs F46C). Fusion callus F46B [(*Tv* 142094 + *Tv* 50) + *Tc* 22] was equally similar to *Tv* 50 and to *Tc* 22 (0.472), but the control mix of tansy and pyrethrum DNA was more similar to pyrethrum (distance 0.245 and 0.292) than to the tansy RAPD pattern (distance 0.373 and 0.417). Fusion callus F43A (*Tv* 50 + *Tv* 142094) was more similar to *Tv* 50 (distance 0.269) than to *Tv* 142094 (distance 0.519) (Table 2).

Average linkage-cluster analysis separated the samples into two groups. Both of the tansy genotypes and the fusion F43A belonged to one group, whereas pyrethrum, fusions F46B, F46C, and artificially mixed DNA from tansy and pyrethrum belonged to the other group (Fig. 2).

Analysis of volatile compounds

Kovat's indices, authentic compounds and the mass-spectral library were used to identify the composition of the dichloromethane extracts. In total 36 volatile organic compounds (KI_{1800}) were quantified, of which 24 were either identified or tentatively identified. No

Table 2 Genetic distance between tansy (*Tv* 142094), pyrethrum (*Tc* 22) and protoplast fusion-derived calli (F43A, F46B, and F46C) based on RAPDs using the method described by Nei and Li (1979)

Source	*Tv* 50	*Tc* 22	*Tv* 142094 + *Tc* 22[a]	*Tv* 50 + *Tc* 22[a]	F43A	F46B	F46C
Tv 142094	0.381	0.714	0.373	0.529	0.513	0.593	0.577
Tv 50		0.762	0.569	0.417	0.269	0.472	0.451
Tc 22			0.245	0.292	0.654	0.472	0.490
Tv 142094 + *Tc* 22[a]				0.179	0.503	0.443	0.458
Tv 50 + *Tc* 22[a]					0.379	0.246	0.254
F43A						0.355	0.333
F46B							0.031

[a] Equally mixed samples of tansy and pyrethrum DNA

compounds in common for the fusion-derived calli (F43A, F46B) and tansy (*Tv* 142094) or pyrethrum (*Tc* 22) were observed. Decadienal, artedouglasia oxide, syringaldehyde, heptadecane and coniferyl alcohol were identified only from protoplast fusion-derived calli (F43A, F46B). Coniferyl alcohol was also found from the protoplast-derived callus of *Tv* 142094. *Beta* farnesene and germacrene D were volatiles present in both tansy (*Tv* 142094) and pyrethrum (*Tc* 22) (Table 3).

Among the less volatile compounds (oven temperature 164–260°C) hexadecanoic and linoleic acid were present in both the protoplast fusion-derived calli (F43A, F46B) and tansy (*Tv* 142094). One unidentified compound (KI = 2264) was detected in the fusion callus (F46B) and pyrethrum (*Tc* 22). In addition, several compounds were observed only from protoplast fusion-derived calli (F43A, F46B). Four peaks, with mass spectra corresponding to those of authentic pyrethrins I (KI = 2281 and 2321) and II (KI = 2264 and 2294), were detected from pyrethrum (*Tc* 22) extracts. Peaks with mass spectra corresponding to pyrethrins were not observed in the protoplast fusion-derived calli (data not shown).

Discussion

Microcolonies and calli derived from the protoplast fusion between tansy and pyrethrum grew more vigorously than the calli derived from the intraspecific tansy fusion. Increased cell growth in the interspecific hybrid callus may be due to heterosis, which was also observed in the hybrids of *L. sativa* × *L. virosa* (Matsumoto 1991) and *Solanum brevidens* × *Solanum tuberosum* (Polgár et al. 1993). In the latter case the vigorous growth was successfully used as a selection marker for hybridity at the callus level. The intraspecific fusion between two tansy genotypes produced very slow-dividing cells lacking green pigmentation, in contrast with the green and rapid-dividing cells of tansy protoplasts (Keskitalo et al. 1995). Slow-dividing cells have been observed in protoplast fusion-derived *Senecio* hybrids which had chlorophyll-deficient spots in the stems associated with a loss of chromosomes or fragments of chromosomes (Wang and Binding 1993). Similarly, abnormal chromosome number was associated with the slow growth of intraspecific *Lactuca* hybrids (Matsumoto 1991).

In our previous study the DNA content for tansy line *Tv* 14 was 7.84 (Keskitalo et al. 1998). *Tv* 142094, which is a sibling of *Tv* 14, had a nuclear content of 6.41 pg in the present study. One reason for the difference in the nuclear DNA content may be that *Tv* 142094 is derived from a pre-mature seed whereas the *Tv* 14 used previously was micropropagated from a shoot tip excised from the field-grown genotype *Tv* 14. Both tissue-type and environmental conditions have been shown to influence nuclear DNA content and especially that of the repetitive sequences (Arnholdt-Schmitt 1993). The DNA content of diploid nuclei of maize (*Zea mays* L.) varied between different plant tissues (Biradar and Rayburn 1993), whereas the 4C DNA content of sunflower (*Helianthus annuus* L.) was observed to vary depending on the site of the seed in the flower from which the regenerants were derived (Natali et al. 1995). Comparable 2C contents were detected from pyrethrum and the protoplast-derived calli, which were analyzed using the same procedure with two different flow cytometers. Interestingly, the chromosome complement 2n = 2C = 18 is the same for tansy (Virrankoski and Sorsa 1968) and pyrethrum (Pal 1992), but the nuclear DNA content of pyrethrum is about twice that of tansy. The nuclear DNA content of the calli derived from the two different types of fusion (tansy + tansy; tansy + pyrethrum) varied, but was more than the parental level in many fusion calli. The increased DNA content suggests that some degree of protoplast hybridization had occurred.

The genetic distance between the two tansy genotypes included in this study (0.381) was comparable to the range of different tansy genotypes observed in our previous study (Keskitalo et al. 1998).

Volatile compounds, such as artedouglasia oxide, syringaldehyde and coniferyl alcohol, were only detected from protoplast fusion-derived calli. This is in agreement with Banthorpe and Brown (1989) who observed that leaf calli of tansy did not contain compounds present in the parental plant but did contain significant concentrations of novel compounds which were rare, or else not detected, in the intact plant. In contrast, small amounts of thujone were observed from a leaf-derived callus of tansy (Svoboda et al. 1995). Growth conditions, such as the type of carbon source and concentration (Banthorpe and Brown 1989; Rajasekaran et al. 1991; Brown et al. 1996), and the presence of hormones (Banthorpe and Brown 1990; Dhar and Pal 1993), have been reported to regulate the accumulation of terpenes in tissue culture-derived calli from *Tanacetum* sp. The small number of volatiles detected from the protoplast-derived calli of tansy and the protoplast fusion-derived calli in this study indicated that the de-differentiated callus seldom produces or accumulates terpenes (Banthorpe et al. 1986; Banthorpe and Brown 1989; Brown et al. 1996). The detection of a precursor compound for pyrethrins, chrysanthemic acid (Kuech et al. 1985; Zito and Tio 1990), was also an indication of the effect of de-differentiated plant tissues on secondary metabolism. Similarly, the low number of compounds found from fusion-derived calli, which are present in both tansy and pyrethrum, may not indicate the absence of hybridization, but rather the inability of non-organized tissue to produce volatile compounds.

The highest DNA content (19.59 pg, range 19.058–20.122) of callus 43C (tansy + tansy) indicates a fusion between three protoplasts. Genetic-distance and cluster

Table 3 Composition of solvent extracted volatile compounds ($R_I < 2000$) from tissue cultured tansy (*Tv* 142094), protoplast-derived calli of tansy (*Tv* 142094), protoplast fusion-derived calli (F43A and F46B), and greenhouse grown pyrethrum (*Tc* 22)

R_I	Compound	Composition (%)[1]				
		Tv 142094 Tissue cultured	*Tv* 142094 Protoplast derived calli	F43A Fusion-derived calli	F46B Fusion-derived calli	*Tc* 22 Greenhouse
937	α-pinene	0.5	–[2]	–	–	–
984	β-pinene	0.6	–	–	–	–
1026	*o*-cymene	0.5	–	–	–	–
1033	FW = 154	0.3	–	–	–	–
1056	2,6-dimethyl-6-nitro-2-hepten-4-one	0.8	–	–	–	–
1141	*trans*-pinocarveol	2.3	–	–	–	–
1148	FW = 152	1.1	–	–	–	–
1164	Pinocarvone	1.4	–	–	–	–
1201	Myrtenol	31.9	–	–	–	–
1230	FW = 152	1.8	–	–	–	–
1298	FW = 152	0.5	–	–	–	–
1315	E,E-2,4-decadienal	–	–	2.2	–	–
1386	FW = 182	1.1	–	–	–	–
1389	FW = 204	0.9	–	–	–	–
1415	β-Caryophyllene	2.4	–	–	–	–
1424	FW = 224	1.0	–	-	–	–
1430	β-Farnesene	1.4	–	–	–	38.0
1462	Unknown	0.7	–	–	–	–
1477	Germacrene D	11.6	–	–	–	20.3
1518	Butylated hydroxytoluene	0.8	–	–	–	–
1528	FW = 204	3.9	–	–	–	–
1523	Artedouglasia oxide	–	–	2.2	–	–
1547	2′-methylacetophenone[3]	2.2	–	–	–	–
1550	4′-methylacetophenone[3]	4.9	–	–	–	–
1584	Caryophyllene oxide	1.8	–	–	–	–
1600	Unknown	–	–	–	–	41.7
1602	Diethyl phthalate	7.1	47.6	20.0	37.8	–
1640	Unknown	2.2	–	–	–	–
1662	Globulol	7.7	–	–	–	–
1663	Syringaldehyde	–	–	–	0.5	–
1668	Khusinol	2.8	–	–	–	–
1676	Unknown	3.2	–	–	–	–
1698	Heptadecane	0.1	–	27.2	–	–
1711	Tetradecanal	–	–	–	2.4	–
1732	1,2-dimethoxy-4-propenylbenzene[3]	–	3.4	–	–	–
1738	E-coniferyl alcohol	–	48.9	43.2	59.3	–

[1] Composition calculated from total ion current of GC-MS analysis
[2] Not detected, or value lower than 0.1%
[3] Tentative identification

analysis suggest that the fusion may have occurred between two protoplasts of *Tv* 50 and one of *Tv* 142094. We arrived at this conclusion because the genetic distance from *Tv* 50 to the fusion callus (F43A) was closer than that from *Tv* 142094, and clustering positioned the fusion callus (F43A) closer to *Tv* 50 than to *Tv* 142094.

The nuclear DNA content (31.87 pg; range 30.315–33.425) measured for callus F46B (tansy + pyrethrum) suggests a fusion between more than two protoplasts, or else a double fusion. Because the average linkage method clustered the F46B callus closer to pyrethrum than to tansy, the fusion probably involved more *Tc* 22 protoplasts. Also the DNA content of F46B is close to the sum of $2 \times$ pyrethrum $+ 1 \times$ tansy. In conclusion, several observations support the occurrence of fusions between tansy and pyrethrum. Firstly, the genetic distances from tansy and pyrethrum to the fusion calli (F46B, C) are smaller than the distance between tansy and pyrethrum. Secondly, the genetic distances from both of the donor species to the calli are almost the same. Thirdly, the artificially mixed sample of tansy and pyrethrum DNA is more similar to the fusion calli than to either tansy or pyrethrum. Difficulties to regenerate the fusion calli, in spite of several attempts, may be a consequence of abnormal chromosome combinations, the loss of chromosomes or chromosome fragments, as observed in other somatic hybrids (Wang and Binding 1993), also partly explaining the variation in the nuclear DNA contents.

Our results show that protoplast fusion between pyrethrum and tansy is possible. Although only a limited differentiation and organogenesis of roots and sporadic shoot formation of tansy has been observed in our previous studies, the regeneration potential evidently exists. However, factors affecting organogenesis, such as the genotype effect on regeneration, still need further investigation.

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