

Research Note

Can protoplast production from *in vitro* cultured shoots of *Tanacetum* vary during the season?

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Two different experiments were carried out to study the production of protoplasts and the variation of protoplast yield from *in vitro* cultured shoot tips of tansy (*Tanacetum vulgare* L.) and pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schiltz-Bip). In the first experiment, light had more pronounced effect for tansy than for pyrethrum. When the donor tissues of tansy were cultured under high light intensity the leaves contained anthocyanin and became brown during enzyme maceration. In contrast, donor tissues cultured under low light intensity produced leaves without anthocyanin. Depending on the light intensity of donor tissues, on average $5.8\text{--}6.8 \times 10^6$ and $3.4\text{--}4.3 \times 10^6$ protoplasts were isolated from one gram of mesophyll leaves of tansy and pyrethrum, respectively. In the second experiment, the production of protoplasts from tansy and pyrethrum varied seasonally. The most successful season for the production of protoplasts from *in vitro* cultured shoot tips was between December and April, when also the highest number of protoplasts could be isolated. It was not possible to state whether *Tanacetum* species have rhythms, which could cause physiological or chemical changes for the *in vitro* grown shoot tips. However, some external or internal, possible seasonal-dependent stimuli may have caused variation in the number of protoplasts isolated from tansy and pyrethrum and favoured protoplast production during winter and spring.

Key words: light, morphogenesis, protoplasts, pyrethrum, sugars, *Tanacetum cinerariifolium* (Trevir.) Schiltz-Bip., *Tanacetum vulgare* L., tansy, tissue culture

Introduction

Development of protoplast techniques has focused mostly on the concentration and type of cell wall degrading enzymes (Chanabe et al. 1989), the source of nitrogen (Guilley and Hahne

1989) and the physical environment (solid, liquid) of the culture medium (Fischer and Hahne 1992). Many experiments have indicated that the source of explants or the developmental state of the explant are also important (Krasnyanski and Menczel 1993, Petitprez et al. 1995, Wingender et al. 1996, Laparra et al. 1997).

Light is the major source of energy for autotrophic growth, and influences physiological, morphological, genetical and chemical mechanisms in plants (Thompson 1991, Delgado et al. 1996, Kloppstech 1997, Spalding 2000). The effects of light on *in vitro* grown shoots and cultured protoplasts can differ from those acting under *in vivo* conditions, and may not always be beneficial. For example, protoplasts, cells lacking a cell wall, are fragile and sensitive to changes in culture conditions. However, there have been relatively few studies on the effects of light on donor tissues used for protoplast isolation (Zhao et al. 1995, Geng-Guang 1996). The effect of light on cell wall structure and chemical composition has been reported however (Parvez et al. 1996). The effect of light may be very complicated and variable even in different tissues and individual cells, as reviewed recently (McClung 2001).

Protoplast techniques have recently been used with two species producing bioactive isoprenoid compounds, namely tansy (*Tanacetum vulgare* L.) (Keskitalo et al. 1995, Keskitalo et al. 1999) and pyrethrum [*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip. syn *Chrysanthemum cinerariifolium* Vis.] (Malaure et al. 1989). To enable the application of genetic and chemical improvement by protoplast fusion (Keskitalo et al. 1999), a large number of protoplasts are required. Previous results indicated that even if the procedure during protoplast isolation was the same, the number of isolated protoplasts varied considerably. We speculated that light might be one of the factors affecting growth of donor tissues and the number of isolated protoplasts (Keskitalo et al. 1995). An other question risen from our previous experiments was that, can protoplast production from *in vitro* cultured shoots of *Tanacetum* vary during the season? Therefore, in this paper we wanted to study the possible effect of light intensity and season on protoplast production from *in vitro* cultured tansy and pyrethrum.

Material and methods

Two different experiments were carried out considering protoplast production and the number of isolated protoplasts per one gram of fresh leaves. For both experiments, the *in vitro* shoot tip culture of tansy and pyrethrum genotypes was done according to Keskitalo et al. (1995) with minor modification. For protoplast isolation, tansy and pyrethrum clones were tissue cultured on MS medium (MS) (Murashige and Skoog 1962) supplemented with 30 g l⁻¹ sucrose, 6 g l⁻¹ agar, and with 1.6 µM NAA (1-Naphthalene acetic acid) (pH 5.8). Cultures were placed under a 16 h photoperiod with illumination from fluorescent lamps (24 ± 2°C / 16 ± 2°C) at 20–80 µM m⁻² s⁻¹.

In the first experiment (light intensity), shoot tip cultures were placed under a 16 h photoperiod with illumination from fluorescent lamps (24 ± 2°C / 16 ± 2°C) at two light intensities (20–40 µM m⁻² s⁻¹ and 60–80 µM m⁻² s⁻¹). These treatments are referred as light 1 and 2, respectively (Table 1). In the second experiment (seasonal effect), shoot tips were cultured as above except that the intensity of light was 60–80 µM m⁻² s⁻¹ (Fig. 1).

Protoplasts for the first experiment (light intensity) were isolated during December to March and for the second experiments (seasonal effect) protoplasts were isolated monthly during one year. For both of the experiments protoplasts were isolated from one tansy (*Tanacetum vulgare*, Tv 14) and three pyrethrum genotypes (*Tanacetum cinerariifolium*, Tc 18, 21, 22) as described previously (Keskitalo et al. 1995, Keskitalo et al. 1999). Leaf tissue was first macerated in enzyme solution (16–22 h) in the dark (29 ± 1°C) with shaking (30 rpm). Digested leaf material was filtered through a nylon sieve and spun to float the protoplasts. An aliquot of solution containing sucrose (0.5 M) and 2-N-Morpholinoethanesulphonic acid (MES) (1 mM) (pH 5.6) was added and the protoplasts were resuspended, and centrifuged. Protoplasts were washed twice.

The weight of the leaves used for the experiments was measured before the enzyme incubation. After the protoplast isolation, the density of protoplasts was determined with a haemocytometer. Therefore, the number of protoplasts isolated in one gram of fresh leaves could be assessed and was referred to as the yield of protoplasts. The viability of the cells was tested using Fluorescein diacetate (FDA) staining. The term 'successful' protoplast isolation was used when protoplasts were released freely from the macerated leaf tissues after the incubation. The statistical difference in the number of protoplasts released between treatments was tested using a *t*-test.

Protoplasts were plated at a density of 3×10^5 cells ml^{-1} in modified MS medium in 5-cm-diameter Petri dishes as described previously (Keskitalo et al. 1995, 1999). Protoplast cultures were solidified two weeks after isolation with modified MS medium, and culture medium was refreshed every week. The concentrations of salts and sugars were gradually changed during the 1–1.5 months of culture to correspond to the concentrations in regular MS medium. Protoplasts were cultured in darkness ($29 \pm 1^\circ\text{C}$) until callus colonies were visible. Small (1 mm \varnothing) calli were transferred to MS medium (agar 6 g l^{-1} ; pH 5.8) supplemented with glucose (30 g l^{-1}), NAA (8.59 μM) and 6-Benzylaminopurine (BAP) (7.10 μM) and placed under a 16 h photoperiod with $40\text{--}200 \mu\text{M m}^{-2} \text{s}^{-1}$ at $24 \pm 2 / 18 \pm 2^\circ\text{C}$.

Results and discussion

All the isolations were carried out within the same year. In the first experiment with two light intensities ($20\text{--}40 \mu\text{M m}^{-2} \text{s}^{-1}$ and $60\text{--}80 \mu\text{M m}^{-2} \text{s}^{-1}$), there were 138 isolations from tansy and 94 from pyrethrum (Table 1). In the second experiment, there were 260 isolations from tansy and 159 from pyrethrum during the entire year (Fig. 1). Overall, in the both experiments,

tansy yielded more protoplasts than pyrethrum ($P < 0.001$). The viability of protoplasts was usually high (80–90%) when a large number of protoplasts were obtained ($> 4 \times 10^6$ from tansy and $> 3 \times 10^6$ from pyrethrum) (data not shown).

Light intensity affected the growth of *in vitro* cultured shoot tips and the production of protoplasts. Tansy shoot tips differed visually depending on the light intensity during *in vitro* culture. Under high light intensity ($60\text{--}80 \mu\text{M m}^{-2} \text{s}^{-1}$) tansy grew slowly, the anthocyanin pigmented leaves were thick, and only a few shoots were produced. In contrast, leaves of tansy grown under low light intensity ($20\text{--}40 \mu\text{M m}^{-2} \text{s}^{-1}$) grew larger and no anthocyanin was detected. Also, browning of leaf tissue during the enzyme maceration was reduced when the shoot tips were cultured under low light intensity. The mean number of isolated protoplasts was 5.8 and 6.8 $\times 10^6$ and 3.4 and 4.3 $\times 10^6$ per single gram of fresh leaves for tansy and pyrethrum, for high and low light intensity, respectively (Table 1). The yield of tansy protoplasts was significantly higher ($P < 0.05$) when isolated from donor tissues cultured under $20\text{--}40 \mu\text{M m}^{-2} \text{s}^{-1}$. The percentage of successful isolations increased by 10% in tansy whereas the percentage for pyrethrum decreased by almost 20% in pyrethrum, when the light intensity decreased (Table 1).

The effect of season on the number of isolated protoplasts is illustrated in Fig. 1. There was a seasonal influence on number of isolated protoplasts. Isolations carried out during the winter (December–February) and the spring (March–May) yielded more protoplasts ($P < 0.05$) compared with the number of isolated protoplasts during summer (June–August) and autumn (September–November). Also, the percentage, referring to the success in isolating protoplasts overall, increased almost linearly with the increase in the number of protoplasts (Fig. 1). The division of tansy cells and the formation of callus were observed to be the highest, when protoplast isolations were performed in February, March, and April. During that time there were a total of 32 successful protoplast isolations from tansy that resulted in callus formation. The underlying

Keskitalo, M. Protoplast production from in vitro cultured shoots of Tanacetum

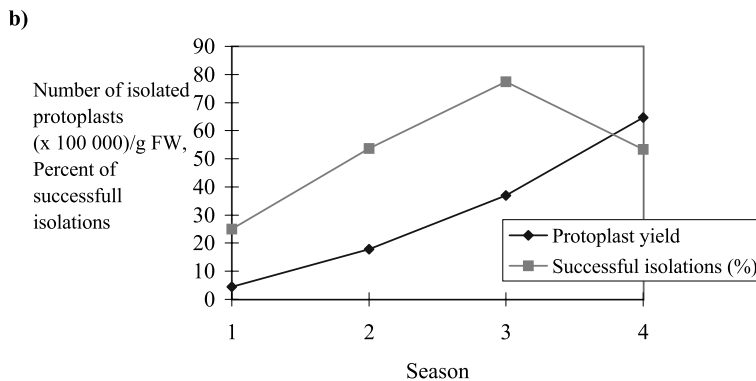
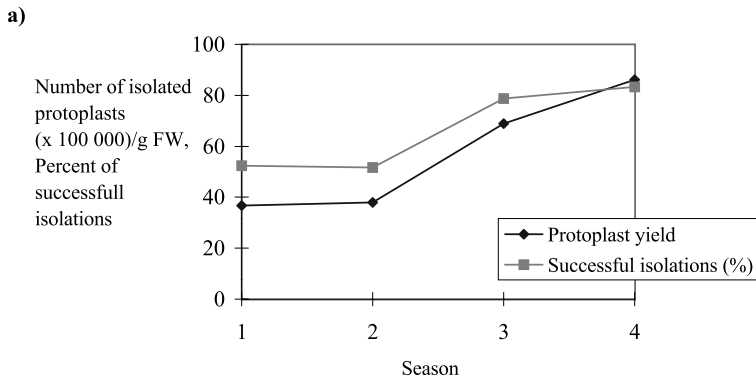


Fig. 1. Effect of season on the number of isolated protoplasts (g^{-1} fresh weight) and on the percent of successful protoplast isolations of tansy (a) and pyrethrum (b). The four seasons are: 1 = summer (June–August), 2 = autumn (September–November), 3 = winter (December–February), and 4 = spring (March–May).

Table 1. Effect of light intensity for the production of protoplasts from tansy and pyrethrum shoot tips.

	Light $\mu\text{M m}^{-2}\text{s}^{-1}$ (treatment)			
	20–40 (1)		60–80 (2)	
	Tansy	Pyrethrum	Tansy	Pyrethrum
Total protoplast isolations	76	26	62	68
Succeeded	59	15	42	51
Percent of successful isolation (%)	77.6	57.7	67.7	75.0
Number of protoplasts/g FW				
Mean	6 800 000	4 300 000	5 800 000	3 400 000
Quantiles of protoplast yield ²				
75%	9 500 000	5 100 000	7 100 000	5 900 000
Median (50%)	4 200 000	2 600 000	3 300 000	1 800 000
25%	2 500 000	1 000 000	700 000	700 000

¹ The intensity of illumination for *in vitro* grown shoot tips

² Quantiles of number of protoplasts / g of fresh leaves. These show the quantiles of 25, 50 and 75% of the observations in the order from the lowest to the highest number of protoplasts.

ing reason may have been the quality or intensity of light. The door of the growth chamber used for callus cultures was occasionally open to improve the ventilation of the room. This may have allowed natural light to supplement the fluorescent lamps. Up to 1% of the isolated tansy protoplasts formed callus and more than 3000 calli per isolation were obtained (data not shown). Pyrethrum protoplasts formed microcalli but their growth did not proceed further. A previous attempt to grow pyrethrum protoplast-derived calli ended at the callus stage (Malaure et al. 1989). Genotypes may differ in their regeneration ability, which should be taken into consideration in future experiments.

Our observations on the positive effect of low light intensity on the yield of isolated protoplasts support results obtained with *Brassica* (Pauk et al. 1991, Zhao et al. 1995) and tomato (*Lycopersicon esculentum* L.) (Bellini et al. 1990) protoplasts. The absence of light was crucial for the division of *Helianthus annuus* L. protoplasts. The frequency of division of protoplasts isolated from seedlings grown in darkness for four days was 1.5–2 times higher than for protoplasts isolated from seedlings grown in the light (Geng-Guang 1996). In *Brassica* twelve cultivars were studied and all produced protoplast-derived callus when the explants were cultured for three days in darkness followed by one day under dim light. In contrast, only six cultivars produced protoplast-derived callus when the protoplasts were isolated from seedlings grown in the light (Zhao et al. 1995). The effect of light was also observed in studies on petunia (Frearson et al. 1973), where the optimum season for protoplast isolation was the same as in our experiments. In addition to the number of protoplasts and the success of protoplasts isolation overall, it would have been very interesting in our experiment to measure the frequency of cell division in greater detail. However, we did not want to disrupt the possible cell division by taking samples from the culture medium.

Light intensity seemed to cause more variation in the number of isolated protoplasts for tansy than for pyrethrum. One of the possible reasons may be that under high light intensity the cells accumulate secondary compounds (Delgado et al. 1996) detected in these species (Keskitalo 1999, Keskitalo et al. 1999, Keskitalo 2001), which may alter the chemical and physical structure of the cell wall (Miyamoto et al. 1994). Secondary compounds can inhibit the function of enzymes or cell proliferation (Parr and Bowell 2000), injuring the plant cell itself. Therefore, shoot tips cultured under high light intensity could contain large amounts of secondary compounds, potentially deleterious to isolated plant cells.

The possible seasonal effect on the number of protoplast yield and on the success of protoplast isolations was observed for both of the species, although the effect of season is difficult to explain. It is known that plants have a circadian rhythm, which is regulated by light and temperature and functions over periods of approximately 24 h. There may be also rhythms that reflect, for example growth rate and hormone production (McClung 2001). Unfortunately, in our experiment we could not exclude factors such as temperature or humidity, which can also cause observed variation in the production of protoplasts. Therefore, whether *Tanacetum* species have rhythms, which could cause physiological or chemical changes for the *in vitro* grown shoot tips or whether the reason was due to other factors we could not exclude, is not possible to state. However, some external or internal and possible seasonal-dependent stimuli may have caused variation in the number of protoplasts isolated from tansy and pyrethrum and favoured protoplast production during winter and spring.

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SELOSTUS

Vaikuttaako vuodenaika *Tanacetum*-lajien *in vitro* kasvuun ja eristettävien protoplastien määrään?

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Valo on elintärkeä yhteyttävälle kasveille ja se vaikuttaa kasvin fysiologiaan, morfologiaan, sekä geneettisiin ja kemiallisiin toimintoihin. Valon vaikutus voi olla kuitenkin erilainen tai jopa vahingollinen solukkoviljelyssä kasvatettujen kasvien kasvuun ja solujen toimintaan. Esimerkiksi protoplastit ovat soluseinättömiä kasvisoluja, joiden avulla voidaan somaattisesti fuusoida risteytymättömiä kasvilajeja kasvinjalostuksessa. Suojaavan seinän puuttuessa protoplastit ovat erityisen herkkiä esimerkiksi valaisuksen muutoksille. Tutkimuksessa käsiteltiin valovoimakkuuden ja vuodenaajan merkitystä kahden *Tanacetum* sukuun kuuluvan kasvin *in vitro* kasvuun ja protoplastien tuotantoon. Pietaryrtin (*Tanacetum vulgare* L.) ja pyrethrumin (*Tanacetum cinerariifolium* (Trevir.) Schiltz-Bip) solukkoviljeltyjen versojen lehtiä käytettiin lähtömateriaalina protoplastien eristämiseen.

Valon voimakkuus vaikutti erityisesti pietaryrtin versojen kasvuun ja protoplastien tuottoon. Voimak-

kaassa valossa kasvatetut versot olivat lyhyempiä ja näyttivät punaisemmilta lisääntyneen antosyaniinin takia. Kun näitä versoja käytettiin lähtömateriaalina protoplastien eristämiseen, protoplastien eristys onnistui harvemmin ja eristettyjen protoplastien määrä tuoretta lehtigrammaa kohti oli pienempi kuin himmeässä valossa tuotetussa lähtömateriaalissa. Protoplastien tuotto pietaryrtillä ja pyrethrumilla vaihteli eri vuodenaikoina. Parhaiten protoplastien eristys onnistui talvella ja keväällä, jolloin myös eristettyjen protoplastien määrä oli suurin. Tutkimus puoltaa aikaisempia havaintoja himmeän valon edullisesta vaikutuksesta protoplastien tuottoon. Vuodenaajan merkitystä solukkoviljelyyn on tutkittu vähän. Tämä tutkimus kuitenkin antoi viitteitä siitä, että myös vuodenaika tai siihen liittyvät tekijät saattavat vaikuttaa *in vitro* kasvatettujen versojen kasvuun ja/tai protoplastieristysmenetelmän onnistumiseen.