REGENERATION OF *PAEONIA MLOKOSEWITSCHII* LOM. AND *P. TENUIFOLIA* L. IN VITRO FROM DIFFERENT EXPLANTS

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ABSTRACT

The pattern of regeneration from tissues of *Paeonia mlokosewitschii* and *P. tenuifolia* cultured in vitro in the same chemical conditions depended on the initial explant. Direct shoot regeneration was obtained from the bases of petioles and petals, and leaf veins. Vegetative initial buds and regenerated in vitro shoots produced on their bases slowly growing nodular callus which was very productive in repetitive shoot regeneration. The tops of stems, flower bases, sepals, petals and ovary walls produced small callus which regenerated white and red spherical structures within 1.5 years. After that time also from those cultures arised nodular, shoot regenerating callus developed.

KEY WORDS: *Paeonia mlokosewitschii*, *Paeonia tenuifolia*, regeneration, nodular callus, flower-like structures.

INTRODUCTION

Herbaceous peonys are attractive perennials with characteristic long juvenile period. Their seeds require up to two years to germinate. Long-lasting conventional breeding and very low propagation coefficient suggest use in vitro methods for both: creation of variability and micropropagation. The precondition for breeding in vitro is knowledge about regeneration of shoots or somatic embryos from individual cells. According to review of Buchheim and Meyer (1992), Radtke (1983) (cit. after Buchheim and Meyer 1992) initial explants of flower buds, pedicels, petioles and stem segments of *P. lactiflora*, produced callus, which failed to regenerate buds or embryos. Lin (1980) (cit. after Buchheim and Meyer 1992) also obtained only callus, and Meyer (1976) (cit. after Buchheim and Meyer 1992) callus and roots. Li et al. (1984) (cit. after Buchheim and Meyer 1992) obtained shoots from the callus produced by axillary buds and petioles of *P. suffruticosa*. Induction of somatic embryogenesis from zygotic embryos of *P. lactiflora* was reported (Buchheim and Meyer 1992) but for breeding and nursery purposes more interesting is the regeneration from adult plants. Our aim was to develop an efficient method for mass plantlet regeneration which could be applied for improvement of *P. mlokosewitschii* (yellow flowers) and *P. tenuifolia* (pink flowers). In this paper we present the pattern of regeneration from different initial explants.

Abbreviations:

BAP – 6-benzylaminopurine, TDZ – 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron), 2iP – 6-(γ,γ -dimethylallylamino) purine, IAA – indole-3-acetic acid, NAA – α -naphthaleneacetic acid, GA₃ – gibberellic acid

MATERIALS AND METHODS

Initiation of cultures

Field-grown shoots at the stage when flower buds were 15-20 mm in diameter served as a source of explants. All possible explant types were isolated from 5 shoots in the first and from 10 shoots in the second year of experiments. These were: vegetative buds from the bases of shoots, bases of petioles, internodes, fragments of leaves with main veins, the highest parts of the shoots being in contact with the flower bud, the bases of flowers, the bases of petals and sepals, ovary walls, pistils and anthers. The number of explants of the given type ranged from 15-30 for vegetative buds to 50-100 for the others. All buds, leaves and stem parts were sterilized separately. They were rinsed for 2 h in tap water, shaken for 30 min in a kitchen detergent, rinsed again in tap water, sterilized for 10 min in 0.1% HgCl₂ and rinsed three times with large amount of sterile distilled water. Finally, they were blotted dry on sterile filter paper, divided into explants and transferred to the medium. The initial medium consisted of 1/2 MS (Murashige and Skoog 1962) mineral salts and vitamins, 30 g/l of sucrose, 250 mg/l milk albumin, 6 g/l Kalys 575 agar and 1 mg/l BAP. After 2 weeks one part of the explants was transferred onto the same fresh medium and another onto medium supplemented with 5 mg/l AgNO₃ for one month. Within the first 1.5 months the cultures were incubated in the dark and then in a growth room at temperatures of 22-25°C and 16/8 h light/dark regime with white fluorescent light of 35 μ mol/m²/s.

Petioles from the shoots regenerated in vitro were also used as a source of explants for regeneration.

Development and growth of structures

All obtained calluses and regenerated structures were transferred every 4-5 weeks onto fresh media consisting of MS salts eventually supplemented with double amounts of CaCl_2 and MgSO_4 . From the sixth month they were subjected to the influence of the following growth regulators: BAP (0.1, 0.5 and 1.0 mg/l), kinetin (0.1, 0.5 and 1.0 mg/l), zeatin (0.5 and 1.0 mg/l), TDZ (0.01 and 0.1 mg/l) or 2iP (1.0 and 5.0 mg/l), IAA (0.1 and 1.0 mg/l) or NAA (0.01 and 0.05 mg/l), GA_3 (0.1, 0.5 and 1.0 mg/l). It was at least 10 explants in each experimental treatment. For histological study calluses and additional structures were fixed in CrAF solution, embedded in paraffin, sectioned into 17 μm slices and stained with safranin, cristal violet and fast green.

RESULTS

Our sterilization method proved to be successful in the first year of experiments when only 10% of initial explants were contaminated. There was no rain for 2 weeks preceding isolation, but next year, when spring was humid, the results were worse. AgNO_3 was helpful in recovery of explants from necrosis. Actually, only explants incubated on the medium containing silver nitrate survived, and callused or regenerated shoots directly.

Direct shoot regeneration was observed only on leaf segments (Fig. 1), bases of petioles and petals already within 2 months at the rate not exceeding 10%. It was not very productive (from 2 to 10 shoots per explant), in addition vitrification and necrosis occurred in almost all these shoots and initial explants. The most effective callus formation was from bases of vegetative buds, and less intensive from sepals, petals, ovary walls, very stem tops and flower bases. Initially, all calluses were white and soft. After 3 months they tended to change; the calluses produced from the vegetative buds hardened and turned yellow, whereas all the others remained soft with necrotic sectors, black film on the surface or turned red. Experiments with growth regulators did not give consistent results. The pattern of regeneration was dependent not on the media composition but rather on the origin of explants. The calluses from vegetative buds grew, divided into hemispheric sectors and regenerated shoots within several months on media containing cytokinin only and cytokinin with 0.1 mg/l IAA or 0.01 mg/l NAA (Fig. 2 and 10). The external and internal structures of calluses resembled nodules (Fig. 9). Arising shoots produced new calluses at their bases which were of similar external and internal structure and high regenerative potential as the original callus. Within 9 months we obtained about 600 shoots from calluses produced on 3 initial non-contaminated vegetative buds. These shoots were capable of elongation, and were mostly not vitrified. The anthers and pistils did not produce callus and died.

The calluses which originated from petioles, detached from shoots obtained in vitro, were soft and reddish and regener-

ated to 50 leafy buds per 5-7 mm callus (Fig. 3), but only about 1/3 of them developed functional meristems and shoots.

The calluses from sepals, petals, tops of the shoots and bases of flowers formed a limited number of spherical structures (from 1 to 5 per callus) – white and red-white-green. Their colour was not dependent on species. The structures were closed with a black "stopper" (Fig. 4 and 11) or they opened at the top forming cup-shaped structures (Fig. 5). Our histological study revealed a free space inside and internal surface-like tissue (Fig. 12). Some structures "burst" and new ones were formed inside (Fig. 13). Some of these spherical structures generated leaf-like rosettes on the media with BAP 0.1 or kinetin 0.5 mg/l (Fig. 6). On the margins of "leaves" new spherical lily-shaped structures were formed (Fig. 14). Explants from the ovary walls produced "hairy", boat-shaped neoformations and inside them a number of spherical structures appeared repeatedly (Fig. 7). Such a pattern of regeneration remained constant throughout a period of 1.5 years. Most of spherical structures died but some changed after that time into nodules and followed a "shooty" pattern of regeneration (Fig. 8).

DISCUSSION

Regeneration of flower buds or structures resembling them from different explants in vitro was reported several times. They regenerated directly from the domes of shoot meristems of *Eleusine coracana* (Wakizuka and Yamaguchi 1995), filament tissue of *Rhododendron* (Shevade and Preece 1993), tepal explants of *Hyacinthus orientalis* (Hosokawa and Fukomaga 1995) or on the callus derived from inflorescence segments of finger millet (George and Eapen 1990), also on the callus from *Rhododendron* filaments (Shevade and Preece 1993), as well as on long term callus cultures derived from *Carthamus tinctorius* cotyledons (Orlikowska and Dyer 1993). The conditions which enable such regeneration pattern were defined long before the quoted papers (review by Scorza 1982). The phenomenon was mostly observed when regenerating were explants originated from generative organs or from stem segments located close to flowers, and on media containing cytokinin. It was clearly demonstrated by Tran Thanh Van (1973) that in cultures from the dermal tissue peeled-off from tobacco stems, the potential to form flower buds depended on the position of the shoot segment from which the tissue originated in a whole plant. When if strips were excised from floral bud branches, 100% of them directly regenerated floral buds. Strips from basal parts of the stem regenerated only vegetative buds. Strips from middle locations developed both, vegetative and flower buds. We obtained a similar pattern of regeneration in *Paeonia* cultures. The nodular calluses (Ito et al. 1996) received from initial vegetative buds which developed on the basal parts of flowering shoots and on petioles of shoots regenerated in vitro, as well as on bases of petioles and leaf segments, regenerated only shoot or leafy

Fig. 1. Direct regeneration from main leaf veins of *P. mlokosewitschii*.

Fig. 2. Regeneration of shoots from nodular callus of *P. mlokosewitschii*.

Fig. 3. Leafy buds growing out of callus originating from leaf petioles derived from shoots of *P. tenuifolia* regenerated in vitro.

Fig. 4. Spherical structure with "stopper" produced on callus from petals of *P. mlokosewitschii*. Bar=1 mm.

Fig. 5. Cup-shaped structure regenerated on callus produced from sepals of *P. mlokosewitschii*. Bar=1 mm.

Fig. 6. "Leaf" rosette regenerated on callus produced from flower base of *P. mlokosewitschii* with spherical structures on margins.

Fig. 7. Spherical structures regenerated from inside of "hairy boat" produced from ovary of *P. tenuifolia*. Bar=3 mm.

Fig. 8. Regeneration of shoots from nodular callus originated from flower base of *P. mlokosewitschii* after 1.5 years culture.

Fig. 9. Structure of nodular callus of *P. mlokosewitschii*. Bar=500 μ m.

Fig. 10. Regeneration from nodular callus of *P. mlokosewitschii*. Bar=500 μ m.

Fig. 11. Histology of the spherical structure with "stopper" from Fig. 4. Bar=500 μ m.

Fig. 12. Spherical structure from Fig. 6 showing internal surface. Bar=500 μ m.

Fig. 13. "Bursting" structure from Fig. 7. Bar=500 μ m.

Fig. 14. Lily-shaped structure from Fig. 6. Bar=250 μ m.

buds. All initial explants excised from flower parts produced calluses which, after some months, regenerated spherical structures resembling flowers or fruits. These eventually transformed into "leafy" rosettes or boat-shaped hairy neoformations on which new generative-like structures appeared. After about 1.5 years nodular calluses which regenerated new shoots very effectively were grown from these structures. The only exception was direct shoot regeneration by petal bases. The pattern of regeneration did not depend on the media used but on the origin of initial explant. It appeared that floral genes expression in *Paeonia* may last for about 1.5 years after separation of explants from the plants. Nevertheless, this conclusion does not apply to other plants because such genera as *Rose* (Arene et al. 1993) or *Chrysanthemum* (Hattori 1992) produce only vegetative buds from flower parts. The choice of the proper explants for effective shoot regeneration is important. It was confirmed that not all parts of a plant are suitable as a source of explants. In both *Paeonia* species the earliest, long lasting and most efficient regeneration of shoots in good condition can be obtained from calluses produced by vegetative buds. Due to the fact that in herbaceous peonys they are located close to the soil, establishing non-contaminated cultures from such buds may be difficult. In that case such flower parts as bases of petals, sepals, ovary walls, flower bases and tops of the stem can provide alternative sources of explants, but their potential to regenerate shoots can be revealed only after 1.5 years.

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REGENERACJA Z RÓŻNYCH EKSPLANTATÓW *PAEONIA MLOKOSEWITSCHII* I *P. TENUIFOLIA* W KULTURACH IN VITRO

STRESZCZENIE

Kultury sterylne zakładano z różnych części pędów *Paeonia mlokosewitschii* i *P. tenuifolia*: pąków wegetatywnych wykształconych na dolnej części pędów, międzywęźli, nasad ogonków liściowych, fragmentów liści z rozgałęzieniem głównych nerwów, wierzchołka pędu, podstawy pąka kwiatowego, nasad działek i płatków kwiatowych, ścian zalążni, fragmentów słupków i z pylników. Rodzaj regeneracji zależał od typu eksplantatu inicjalnego. Bezpośrednią regenerację pąków liściowych otrzymano z nasad ogonków liściowych, nerwów liściowych i płatków kwiatowych. Pąki wegetatywne inicjalne i regenerujące w warunkach in vitro pędy tworzyły u podstawy wolno rosnący kalus, przekształcający się w nodularny, który bardzo wydajnie i powtarzalnie regenerował pędy. Wierzchołek pędu, podstawa pąka kwiatowego, nasada płatków i działek kielicha oraz ściany zalążni tworzyły mały, luźny kalus, który regenerował białe i czerwone sferyczne struktury kwiatowo- lub owocopodobne przez około 1,5 roku. Po tym czasie także z tych kultur tworzył się kalus nodularny, masowo regenerujący pędy.

SŁOWA KLUCZOWE: *Paeonia mlokosewitschii*, *P. tenuifolia*, regeneracja in vitro, kalus nodularny, struktury kwiatopodobne.