PLANT REGENERATION FROM IMMATURE EMBRYO CULTURE OF *TRIFOLIUM MICHELIANUM* SAVI. – HISTOLOGICAL OBSERVATIONS ON ADVENTITIOUS SHOOT INDUCTION

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ABSTRACT

Whole plant regeneration via organogenesis in immature embryo culture of *Trifolium michelianum* Savi. was obtained. The shoots were induced directly from the hypocotyl or indirectly through callus on EC6 medium supplemented with 0.5 and 5 mg/l BAP, respectively. Sporadically the callus formation was preceded by shoot regeneration. Both shoots obtained directly and via callus regenerated into plants when subcultured on half-strength Murashige and Skoog (MS) medium, free of growth regulators. Histological observations revealed that the shoots were induced directly from the ring of meristematic tissue which was formed at the periphery of the hypocotyl or indirectly from the superficial cells of the callus. Irrespective of the mode of regeneration the shoots were of multicellular origin.

KEY WORDS: *Trifolium michelianum*, Leguminosae, shoot organogenesis, plant regeneration.

INTRODUCTION

Under *in vitro* conditions the adventitive organs can be induced either directly on the original explant (direct organogenesis) or indirectly i.e. on callus tissue (indirect organogenesis) (Hicks 1980). For use in agronomy as well as in studies on plant organ growth and differentiation the direct mode of regeneration has an obvious advantages over the indirect mode. That main advantages are: the short time of induction of organogenesis and the avoidance of the genetic and physiological instability accompanying the callus cultures (Bozzini 1980).

For plants of economic interest such as pasture clovers many systems of whole plant regeneration via shoot organogenesis *in vitro* were described. In most of them however, the adventitious shoots occurred in the presence of callus: *T. repens* (Peletier and Peletier 1971, Gresshoff 1980), *T. alexandrinum* (Mokhtarzedeh and Constantin 1978), *T. incarnatum* (Beach and Smith 1979), *T. pratense* (Phillips and Collins 1979, Oelck and Scheider 1983, MacLean and Nowak 1989, Cebat et al. 1990a), *T. resupinatum* (Oelck and Scheider 1983), *T. alpestre* (Parrott and Collins 1982/83), *T. rubens* (Parrott and Collins 1982/83) and *T. nigrescens* (Konieczny 1995). In contrast, the direct shoot regeneration was obtained only for *T. medium* (Choo 1988), *T. pratense* (Skucińska and Miszke 1980, Cebat et al. 1990b) and *T. repens* (Bond and Webb 1989, White and Voisey 1994). Except for the studies of Cebat et al. (1990a, b) and White and Voisey (1994) no

histological observations dealt with the process of adventitious shoots induction in this genus are available. Such studies however, are very helpful for understanding the mechanism of growth regulators action leading to organ formation.

Preliminary experiments performed in our laboratory (data not shown) revealed that immature zygotic embryos of *Trifolium michelianum* Savi. (balanse clover) responded to BAP stimuli with rapid direct and indirect shoot regeneration. Since immature embryos have not been used for induction of shoots in tissue culture of clovers so far, it was interesting to test the possibility of plant regeneration from such explants. Furthermore the previous study of Webb et al. (1987) found in balanse clover that callus derived from seedling explants produced shoots with low frequency but whole plant regeneration was not reported.

The present paper describes a method for direct and indirect plant regeneration via shoot organogenesis in the culture of immature embryos of *T. michelianum*. To determine the localization of the competent cells involved in the process of shoot regeneration histological observations under light microscopy were undertaken.

MATERIAL AND METHODS

Seeds of *T. michelianum* Savi. were obtained from Insitute für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany. The seeds were sown to plastic pots with

substrate containing soil, sand and perlite (1:1:1, v/v) and incubated in a growth chamber at 25°C with 16h photoperiod for 8 weeks. After this time the plantlets were transferred to soil in the experimental plot.

Pods were collected from open-pollinated flowers and surface sterilized by immersion for 70 sec in 70% (v/v) ethanol, 10 min in 5% (w/v) solution of sodium hypochlorite and rinsed four times in sterile distilled water. Zygotic embryos ranging in size 4-6 mm were excised from the ovules and placed onto EC6 medium (Maheswaran and Williams 1984) without yeast extract supplemented with 0.05, 0.5, and 5 mg/l BAP. The media were solidified with 0.7% (w/v) agar (BBL) and adjusted to pH 5.8 with 1N sodium hydroxide and/or 1N hydrochloric acid before autoclaving. Twenty explants, with 10 per petri dish (100 x 20 mm) were cultured on each medium. The culture was maintained at 25°C with 16h photoperiod provided by cool, white fluorescent light of intensity 700-800 lx.

Adventitious shoots ca 3 cm in length arising either directly on the explants or on calli were removed and transferred to MS (Murashige and Skoog 1962) medium with macronutrients reduced by a half, without growth regulators. After two months regenerated plants with well differentiated roots were transplanted to pots with mixture of soil, sand and perlite (1:2:1, v/v) in a growth chamber.

The quantitative data of shoot organogenesis were recorded at the 14th day of culture.

Histological studies were made for explants cultured on EC6 with 0.5 and 5 mg/l BAP. The samples were collected after 1, 3, 4, 5, 7, 9, 11 and 16 days of culture and fixed with FAA (formalin, glacial acetic acid, 50% ethanol, 5: 5: 9, v/v/v) for 72h, dehydrated in a graded ethanol series and embedded in paraffin. Transverse and longitudinal sections 10 µm thick were cut on a rotary microtome and stained with Haidenhain's haematoxylin or Feulgen technique (O'Brien and McCully 1981). The latter were also counterstained with alcian blue or fast green. The embryos before placing onto culture media were used as a control.

RESULTS

Plant regeneration

The BAP at concentration of 0.05 mg/l was ineffective for induction of shoot organogenesis. Within 2 weeks of culture on this medium the embryos developed into rooted seedlings with one or two leaves.

Fig. 1. Direct shoot regeneration from hypocotyl of developing embryo.

An increase of the cytokinin concentration in the medium to 0.5 mg/l resulted in direct shoot regeneration from hypocotyl region of developing embryos (Fig. 1). The shoots became visible after 6-7 days of culture on 35% of explants with high frequency (on average 18 shoots per embryo were regenerated). They grew rapidly and within 4-6 days formed trifoliate leaves. Till the 9th day of culture all of the shoots induced by 0.5 mg/l BAP arose in the absence of visible callus. After this time little callusing of the original hypocotyl occurred. However, the callus obtained on this medium did not show any sign of morphogenesis and soon necrosed.

Newly formed adventitious shoots ca 3 cm in length were removed from the embryos and transferred to half-strength MS, free of growth regulators for propagation. Since rhizogenesis occurred simultaneously with shoot multiplication no special medium for rooting was required. After two months approx. 80% of adventitious shoots obtained directly from the initial explants regenerated into young plantlets. When transplanted to pots in a growth chamber they established as a mature plants.

In the presence of 5 mg/l BAP the embryo development was always disturbed. The most often the considerable thickening of the embryonic axis and/or the inhibition of primary leaf development were observed (Fig. 2). After 8-10 days of culture on medium with 5 mg/l BAP the hypocotyl of some implanted embryos started producing green and glossy callus. It grew very slowly and at the end of the second week of culture the callus covered with a thin layer only the hypocotyl part of the embryos. Following the next 3-5 days the callus gave rise to shoots (Fig. 2). In comparison with those obtained on medium with 0.5 mg/l BAP they were often vitreous and grew more slowly.

Fig. 2. Differentiation of shoots from callus.

Occasionally on medium containing 5 mg/l BAP the callogenesis was preceded by direct shoot regeneration. In this case the adventitious shoots were formed from significantly swelled embryos within 6-8 days after culture followed by the proliferation of callus with high organogenic potential. Finally 40% of the embryos explanted on EC6 supplemented with 5 mg/l BAP produced callus with 23 visible shoots, on average.

The procedure for multiplication and rooting of callus-derived shoots was the same as described for those formed directly from the original explants. In a result ca 20% of shoots from callus induced on EC6 with 5 mg/l BAP were regenerated into plants (Fig. 3).

Fig. 3. Regenerated plant one month after transplantation to soil.

Histological observations

Since the adventitious shoots occurred on the hypocotyl of the explanted embryos the histological studies focused only on this region.

The hypocotyl epidermis of the control embryos was a single layer consisting of small, isodiametric cells surrounding the cortex of 6-7 layers (Fig. 4). The cortical cells were equal-sized with densely stained cytoplasm and large, centrally positioned nuclei. They were tightly packed with small intracellular spaces. The central part of the hypocotyl was occupied by vascular cylinder consisting of immature xylem and phloem. The pericycle was difficult to distinguish. The cell divisions in the hypocotyl of the control embryo were not observed.

Fig. 4. Transverse section of the embryo hypocotyl at the time of explanting, x135.

After 1 day of culture on medium supplemented with 0.5 mg/l BAP the first cell divisions in the subepidermal layer of the cortex were induced. They were predominantly in periclinal plane and resulted in the groups of small, intensely staining cells clearly visible at the 3rd day of culture (Fig. 5). Following the next 1-2 days the hypocotyl epidermis became more meristematic in appearance and together with cells of the outer cortex formed a compact ring of meristematic tissue at the periphery of the hypocotyl (Fig. 6) along its total

Fig. 5. Longitudinal section of the hypocotyl showing the occurrence of the groups of meristematic cells in subepidermal layer of the cortex, x135.

Fig. 6. Transverse section through the hypocotyl showing the ring of meristematic tissue at the periphery, x135.

length. During this period of culture the cells of the inner layers of the cortex enlarged markedly when compared to the control ones and their nuclei became faintly stained or even invisible. After 5-6 days of culture the mitotic activity in the outer regions of the meristematic ring led to formation of a dome-shaped swellings (Fig. 6) which gradually developed into shoot buds (Figs 7, 8). Well differentiated shoot meristems with defined tunica and corpus formed vascular connections to the main vascular strand of the embryo.

Supplementing the medium with 5 mg/l BAP resulted in induction of mitosis both in the outer and inner regions of the cortical parenchyma (Fig. 9). After 4-6 days of culture the resulting masses of small meristematic cells localized in the internal region of the cortex (Fig. 10) gave rise to an endogenous callus whereas those localized at the periphery of the hypocotyl differentiated into shoot buds (Fig. 11). At this stage the epidermis of the hypocotyl was still continuous with the epidermis overlying the newly formed adventitious buds. After 10 days of culture the rapid expansion of the endogenous callus led to the rupture of the epidermis and outgrowth of the callus over the explant surface. Soon after the shoot meristems arose from the peripheric regions of the calli (Fig. 12).

Fig. 7. Longitudinal section of the hypocotyl showing well differentiated shoot bud, x135.

Fig. 10. Transverse section of the hypocotyl showing the formation of meristematic tissue in the inner region of the cortex, x150.

Fig. 8. Transverse section of the hypocotyl showing numerous meristemoids at the periphery, x135.

Fig. 11. Transverse section of the hypocotyl showing the development of the endogenous callus accompanying shoot bud formation, x100.

Fig. 9. Transverse section of the hypocotyl showing the induction of mitosis in the outer and inner region of the cortex, x275.

Fig. 12. Meristemoid on the surface of the callus, x135.

BAP at the concentration used in this study did not induce any anatomical changes within the vascular cylinder of the explanted embryos (Fig. 10).

DISCUSSION

The results described in this paper are the first report on plant regeneration in *in vitro* culture of *Trifolium michelianum* Savi. (balanse clover). They revealed that it was possible to induce shoot organogenesis using immature zygotic embryos of balanse clover on medium containing BAP as the only growth regulator. Previously in tissue culture of *Trifolium* only White and Voisey (1994) found this cytokinin to be sufficient to bring about shoot regeneration. In other studies the media containing various auxins like NAA, IAA or picloram in addition to BAP were applied (Phillips and Collins 1979, Choo 1980, Skucińska and Miszke 1980, MacLean and Nowak 1989, Cebrat et al. 1990a,b). However BAP alone was successfully employed for induction of shoots in several legumes e.g. *Vigna aconitifolia* (Eapen and Gill 1986), soybean (Wright et al. 1986), lentil (Polanco et al. 1988), and *Vigna radiata* (Mendoza et al. 1993).

In this experiment the concentration of the cytokinin in the medium had an obvious effect on the course of plant regeneration. Similarly to the study of White and Voisey (1994) BAP at level of 0.5 mg/l was found to be effective for induction of direct organogenesis. In culture of balanse clover however the shoots could be also obtained from callus when concentration of the cytokinin in the medium was increased to 5 mg/l. The occasional occurrence of organs before callusing could be due to the individual differences in organogenetic potential among the embryos. Although the frequency of shoots obtained on media with 0.5 and 5 mg/l BAP was similar, the cytokinin at the highest concentration used (5mg/l) significantly reduced the frequency of plant regeneration. The reasons for this were probably the abnormalities in shoot morphology observed in culture on such medium.

Histological observations revealed that in the hypocotyl of the implanted embryos only cortex and epidermis were target sites for BAP action. The induction of mitosis as early as after one day of culture indicate that at the time of explanting the cells of the cortex could be already competent to respond to the cytokinin stimuli. In legumes, comparable data on the rapid onset of organogenesis induced by BAP has been recently provided by Mendoza et al. (1993) in culture of *Vigna radiata*.

The callus obtained in this study was formed by the multiplication of cells within the cortical parenchyma. However Cebrat et al. (1990a) observed in hypocotyl of red clover that callus formation was mainly associated with the proliferation of the pericycle. Furthermore these authors reported that the cells located deep in the callus underwent differentiation and formed shoots, whereas in calli of *T. michelianum* the buds were always regenerated from the superficial cells. Also the shoots induced directly from the embryos of balanse clover were formed regularly exogenously. They resulted from the ring of meristematic tissue which was produced at the periphery of the hypocotyl showing a close similarity with direct shoot regeneration in *Phaseolus vulgaris* (Franklin et al. 1991). In that study however the formation of the meristematic ring was limited to the nodal region of the embryonic axis, whereas in embryos of *T. michelianum* it was extended along the whole length of the hypocotyl.

All the shoots obtained in this study were produced by simultaneous divisions of several cells and therefore were multi-

cellular in origin. In literature no data on the number of cells involved in shoot formation from callus of *Trifolium* are available. Considering the direct organogenesis, White and Voisey (1994) found in white clover that the shoots were induced from single epidermal cells. This discrepancy in respective results possibly could be due to the species variation or differences in culture conditions.

The exogenous origin of shoots in culture of immature embryos of *T. michelianum* indicate that the competence of cells for organ formation could be determined by their position within the explant. Support for this suggestion is an observation that the cells of the inner layers of the cortex although activated on medium with 5 mg/l BAP did not show any organized growth and gave rise to endogenous callus. The reasons for such differences in organogenetic capacity between the inner and outer regions of the explants are difficult to explain. Thorpe (1980) suggested that they may reflect the differences in the physiological gradient of the substances from the medium into the tissues. The activation of peripheral regions of the explants for direct and indirect shoot differentiation was commonly observed in culture of other legumes e.g. *Vigna aconitifolia* (Eapen and Gill 1986), *Pisum sativum* (Tetu et al. 1990), *Trifolium pratense* (Cebrat et al. 1990b), *Phaseolus vulgaris* (Franklin et al. 1991), *Vigna radiata* (Mendoza et al. 1993), and *Trifolium repens* (White and Voisey 1994).

The described system for plant regeneration via organogenesis in *T. michelianum* is distinct from those reported earlier in clovers since immature embryos were used as explants. Previously in tissue culture of *Trifolium* the whole immature embryos were successfully employed only by Maheswaran and Williams (1984) for regeneration of plants of *T. repens* through somatic embryogenesis.

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REGENERACJA ROŚLIN W KULTURACH *IN VITRO* NIEDOJRZAŁYCH ZARODKÓW *TRIFOLIUM MICHELIANUM* SAVI. – OBSERWACJE MIKROSKOPOWE NAD INDUKCJĄ PĘDÓW PRZYBYSZOWYCH

STRESZCZENIE

Opracowano metodę regeneracji roślin *Trifolium michelianum* Savi. na drodze organogenezy. Pędy przybyszowe indukowano w hodowli niedojrzałych zarodków na pożywce EC6 uzupełnionej 0.5 i 5 mg/l BAP. W czasie kultury na pożywce zawierającej 0.5 mg/l cytokininy pędy indukowano bezpośrednio z hypocotyli implantowanych zarodków natomiast w obecności 5 mg/l BAP zarodki wytwarzały kalus, który następnie regenerował pędy. Niekiedy wytworzenie kalusa poprzedzone było regeneracją pędów bezpośrednio z hypocotyli zarodków. Zarówno pędy otrzymane bezpośrednio jak i te indukowane na kalusie regenerowały w rośliny po przeniesieniu na pożywkę MS z połową zawartości makroelementów. Obserwacje mikroskopowe nad regeneracją pędów wykazały, że pąki przybyszowe były pochodzenia wielokomórkowego i różnicowały się z tkanki merystematycznej powstałej na obwodzie hypocotyli lub z komórek na powierzchni kalusa.

SŁOWA KLUCZOWE: *Trifolium michelianum* Savi., *Leguminosae*, pędy przybyszowe, regeneracja.