ORGANOGENESIS DURING PLANT REGENERATION IN COTYLEDON CULTURE OF *TRIFOLIUM MICHELIANUM* SAVI.

ROBERT KONIECZNY

Department of Plant Cytology and Embryology
Institute of Botany, Jagiellonian University
ul. Grodzka 52, 31-044 Krakow, Poland

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ABSTRACT

Shoots of *Trifolium michelianum* Savi. could be induced directly from the cotyledonary petiole or through petiole-derived callus on EC6 medium (Maheswaran and Williams 1984) supplemented with BAP at 4.4, 8.8 and 22.2 μM . The mode of caulogenesis highly depended on cytokinin concentration in the medium. Most of the shoots regenerated into mature plants when transplanted to half-strong MS (Murashige and Skoog 1962). The time of caulogenic determination was set for the first day of culture. The induction of determination for caulogenesis was accompanied by the loss of competence toward root formation. SEM studies revealed that caulogenesis was restricted to the distal end of the cotyledonary petioles, very close to but not at the site of excision. The sites of shoot regeneration were limited to abaxial and lateral surfaces of the petioles.

KEY WORDS: determination, Leguminosae, organogenesis, plant regeneration, *Trifolium*.

INTRODUCTION

It is generally believed that the high level of auxin to cytokinin in the medium favours root development, whereas the reverse gives rise to shoots. Despite of numerous reports confirming these observations there is still a lot of data inconsistent with this hypothesis (e.g. Walker et al. 1979; Hachey et al. 1991; Lo et al. 1997a, b). Furthermore the mechanism underlying the processes of shoot versus root formation after appropriate phytohormone treatment remains rather poorly understood. In order to give a background for further studies on organogenesis in vitro Christianson and Warnick (1983, 1985) divided this process into the developmental events of competence, induction and determination. According to these authors, the induction of determination can be seen as a change in the fate or destiny of explant cell or group of cells resulting in a specific pathway of development. Since determination state is stable (Christianson and Warnick 1983, 1985) it can be easy to establish in tissue culture: the determined explants will produce organs even after the exposition to contrastive inductive stimuli.

Recently, the time frame for caulogenic determination as well as the anatomical changes related to induction of determination were studied in *T. michelianum* (Konieczny 1996b). In that study, however only shoots could be obtained. Thus the investigations on morphogenic capacity of *T. michelianum* were expanded to find a new experimental system in which both shoot and root organogenesis could be induced by changing in vitro conditions.

This study was designed to elucidate the temporal frame of caulogenic and rhizogenic determination during plant regeneration in cotyledons culture of *Trifolium michelianum* Savi. (balanse clover). Additionally, the quantitative data were supplemented with scanning electron microscopy observations on adventitious shoot formation.

MATERIAL AND METHODS

Explant preparation

Seed source, conditions for donor plant growth and pods sterilization were described elsewhere (Konieczny 1996a). Cotyledons including petiole were excised from zygotic embryos (3-4 mm in length) and placed adaxial side down on the surface of the media.

Culture media and culture procedure

The basic media used in the experiment were EC6 (Maheswaran and Williams 1984) without yeast extract and MS (Murashige and Skoog 1962) with reduced by a half macro- and microelements. The media were supplemented with 0.7% and 0.8% (w/v) BBL agar, respectively and adjusted to pH at 5.7 using 1N NaOH and/or 1N HCl before autoclaving.

For plant regeneration, the cotyledons were cultured on EC6 supplemented with 2.2, 4.4, 8.8 and 22.2 μM BAP. After 14 days the percentage of the explants producing shoots and/or callus as well as the mean number of shoots produced per explant were counted. At the same time the adventitious shoots about 3 cm in length were removed from the original

explants and rooted on half-strength MS. Twenty five shoots from each treatment were incubated in five 250 ml Erlenmeyer flasks. After 30 days the frequency of plant regeneration (percentage of shoots regenerated into plants) was determined. Regenerated plants were transplanted to soil and maintained under the conditions described earlier (Konieczny 1996a).

For studying the onset of caulogenic determination the cotyledons were cultured on EC6 containing BAP at 4.4 μM for one, three and five days and then transferred to EC6 hormone-free medium. To determine the ability of the explants to regenerate roots the cotyledons were explanted directly on EC6 basic medium and cultured for 14 days. Quantitative data of rhizogenesis and caulogenesis (percentage of the explants producing shoots or roots as well as the mean number of shoots regenerated per explant) were recorded after 14 days of culture.

Cultures were incubated at 25°C with 16h daylength of white fluorescent light at approximately 100 μM photons $\text{m}^{-2}\text{s}^{-1}$.

The experiments were designed as randomized complete blocks with five independent replicates. Each replicate Petri dish contained five cotyledons with petiole. Analysis of variance was done for each treatment. Statistically significant mean differences were determined by means of t-Student's test.

Scanning electron microscopy

For microscopic studies the cotyledons were taken after one, three and five days of culture on EC6 supplemented with 4.4 μM BAP. Material was fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH=7.2) at room temperature for 2 h and then postfixed in 2% aqueous OsO_4 for 12 h at 4°C. After dehydration in a graded ethanol series the samples were critical-point dried, covered with gold and photographed with a JEOL JSM 54-10 microscope at 25 kV. The cotyledons excised from the embryos before placing on the culture medium were used as a control.

RESULTS

Plant regeneration

The mode of shoot regeneration from cotyledonary petioles of *T. michelianum* highly depended on BAP concentration in the medium (Tab. 1). Direct shoot formation was obtained when EC6 containing 4.4, 8.8 and 22.2 μM of the cytokinin were used (Fig. 1a). Additionally callus formation and subsequent shoot regeneration on media with 8.8 and 22.2 μM BAP were found (Fig. 1b).

The optimal BAP concentration for direct caulogenesis was estimated as 4.4 μM (Tab. 1). The increase of the cytokinin in the medium up to 22.2 μM markedly reduced the frequency

of direct caulogenesis, however did not influence significantly the mean number of shoots regenerated per explant. The concentration of BAP had no effect on the time of induction of direct caulogenesis; in all treatments the shoots became visible already after five to six days after culture.

In cultures with 8.8 and 22.2 μM BAP abundant callogenesis occurred (Tab. 1). Callus proliferation began after four to six days of culture at the cut ends of the petiole. A few days later compact and green callus developed from the whole of the explant including both petiole and cotyledon. However the callus covering the cotyledon did not show any caulogenic potential and the shoots developed only from the uppermost surface of the petiole callus within 10-14 days of culture (Fig. 1b).

Except of shoots obtained in the presence of 22.2 μM BAP all the organs were vigorous and normal. After transferring to half-strength MS they elongated and rooted rapidly giving rise to plantlets (Fig. 1c). In contrast, the shoots induced with the highest concentration of cytokinin (22.2 μM) were often abnormal and mostly failed to develop further despite the transfer to fresh half-strength MS. Detailed data concerning the frequency of plant regeneration are summarized in Table 1.

The lowest BAP concentration used in this study (2.2 μM) did not give rise to callus or shoots.

SEM observations

SEM images of the control explants showed that epidermal cells of the cotyledons and petioles were of rectangular shape and similar size (Fig. 2a). No differences in cell size and cell shape between adaxial and abaxial epidermis of the explant were found. The first signs of morphogenesis were observed after one day of culture. At this time the petioles became swollen when compared with the control, and the large, oval or curved cells at the sites of excision occurred (Fig. 2b). During further culture the number of these cells did not increase and most of them died. During the next two days the distal end of the petioles became nodular in appearance (Fig. 2c). Numerous dome-shaped swellings were always arranged in a more or less complete row along the abaxial and lateral surfaces of the petioles, very close to, but not at the cut surface. With further culture the swellings continued to enlarge forming shoot meristems. The rupture of the original epidermis was not observed so the newly formed meristems maintained the epidermis of the petiole (Fig. 2d). The development of the adventitious shoot was observed after five days of culture. Until this time the shoot formation was restricted only to the distal end of the petioles.

Effect of BAP exposure

When cultured on EC6 lacking BAP induction of rhizogenesis from the distal end of the cotyledonary petiole was ob-

TABLE 1. Effect of BAP concentration on the course of caulogenesis and plant regeneration in cotyledon culture of *T. michelianum*¹.

BAP [μM]	Directly from the explant			Through the callus			
	caulogenesis [%]	mean no. of shoots	plant regeneration [%]	callus [%]	caulogenesis [%]	mean no. of shoots	plant regeneration [%]
0	0	0	0	0	0	0	0
2.2	0	0	0	0	0	0	0
4.4	80c	10.1b	96b	0	0	0	0
8.8	64b	7.3	92b	12a	12a	14.3a	76b
22.2	8a	11.5b	24a	80b	60b	15.2a	28a

¹treatments sharing the same letter are significantly not different at P=0.05 according to t – Student's test.

Fig. 1. Shoot and root development in cotyledon culture of balanse clover.

(A) Direct shoot regeneration on EC6 with 4.4 μ M BAP.

(B) Shoot induction on petiole-derived callus on EC6 with 22.2 μ M BAP.

(C) Young plants growing in soil.

(D) Root induction on EC6 basal medium.

(E) Root and shoot development on EC6 basal medium after one-day exposure to 4.4 μ M BAP.

(F) Secondary root formation after ten days on EC6 basal medium.

served (Fig. 1d). The roots were produced on most of the explants (Fig. 3) within four to six days after culture initiation. Following the next two or three days secondary and tertiary roots formation was noticed (Fig. 1f). Rhizogenesis occurred also after one-day exposure to BAP however, the frequency of root induction was significantly lower when compared with the medium without cytokinin (Fig. 3). Additionally, after one-day treatment of BAP rhizogenesis was accompanied by shoot formation (Fig. 1e). Similarly to the roots, all the shoots were

regenerated regularly from the distal end of the cotyledonary petiole. Increasing the culture period on medium with BAP up to three days resulted in a significant enhancement of caulogenesis and complete inhibition of rhizogenesis (Fig. 3). Both the frequency of caulogenesis and average number of shoots regenerated per explant recorded in this treatment were nearly twice more than those obtained during one-day exposition to BAP. Further prolongation of exposure to cytokinin up to five days had no marked effect either on the frequency of

Fig. 2. Scanning electron micrographs of direct shoot regeneration in culture of balanse clover on EC6 with 4.4 μM BAP.

- (A) Abaxial surface of the cotyledonary petiole before culturing.
 (B) Enormously enlarged petiole with large, irregular cells at the cut surface.
 (C) Meristematic nodules just at the site of excision.
 (D) High magnification of (C). Note the continuity of epidermis.

caulogenesis or the mean number of shoots regenerated per explant. However in explants cultured on BAP for five days little callusing at the shoot base was observed. This callus grew very slowly and did not show any morphogenic potential.

DISCUSSION

Plant regeneration in cotyledon culture of *Trifolium michelianum* Savi. (balanse clover) was obtained. The shoots could be induced directly from the cotyledonary petioles or indirect-

ly through petiole-derived calli when cultured on EC6 (Mahe-swaran and Williams 1984) containing BAP at 4.4, 8.8 or 22.2 μM . Similarly to the culture of zygotic embryos of balanse clover (Konieczny 1996a) the mode of shoot regeneration from the cotyledonary petioles highly depended on cytokinin concentration in the medium. Furthermore, the amount of BAP used for induction of an appropriate mode of regeneration was also similar to that found previously (Konieczny 1996a). In that study however, the frequency of caulogenesis was much more lower than obtained here. High efficient shoot regeneration from the cotyledonary petioles and/or co-

Fig. 3. Effect of time of culture on EC6 with 4.4 μ M BAP on roots and shoots formation from cotyledonary petiole of *T. michelianum*. Treatments sharing the same letter are significantly not different at $P=0.05$ according to t-Student's test.

yledonary node region of the explants was also obtained in other legumes e.g.: *Glycine max* (Wright et al. 1986), *Vigna mungo* (Gill et al. 1987), *V. radiata* (Mendoza et al. 1993), *Phaseolus vulgaris* (Franklin et al. 1995), and more recently *Arachis hypogea* (Kanyand et al. 1997). Within the genus *Trifolium*, however, comparable observations were provided only by White and Voisey (1994) in culture of *T. repens*. In other studies the shoots could be induced from a variety of explants, out of which the hypocotyls (Mokhtarzadeh and Constatntin 1978; Beach and Smith 1979; MacLean and Nowak 1989; Cebrat et al. 1990a; Konieczny 1995), cotyledons (Phillips and Collins 1979; Konieczny 1995), leaves (Oelck and Scheider 1983; Choo 1988), flower heads (Skucińska and Miszko 1980; Cebrat et al. 1990b) and zygotic embryos (Beattie and Garrett 1995; Konieczny 1996a) were used the most often.

SEM images of balanse clover revealed that caulogenesis was restricted to the distal end of the petioles very close to, but not, at the site of excision. This observation concurs with the studies of Mendoza et al. (1993) and White and Voisey (1994). In *T. michelianum*, however, all the shoots resulted from the abaxial and lateral surfaces of the petioles, whereas in *Vigna radiata* (Mendoza et al. 1993) and *T. repens* (White and Voisey 1994) they were regularly formed on the adaxial side. The reasons for these discrepancies are difficult to explain, especially that in the studies mentioned above the cotyledons were explanted in the same manner as in balanse clover: adaxial side touching the medium. In *Vigna*, however, Mendoza et al. (1993) identified the pre-existing meristematic tissue just beneath the adaxial epidermis which was found to be the site of shoot induction. Similar meristematic tissue responsible for shoot regeneration was also described at the cotyledonary base of *Glycine* seedling (Wright et al. 1986). Thus, the histological studies on balanse clover would be very helpful to find the reasons of shoot induction from the abaxial surface of the petioles. According to the data obtained here the abaxial and adaxial epidermis of the petioles were of the same structure with no detectable sites for shoot regeneration. In contrast, in numerous plant species shoot induction was associated with the various epidermal appendages as flower tri-

chomes (Koiwai and Matsuzaki 1988), petals (Miller et al. 1991), glandular hairs (Ohki 1994) or epidermal nodules (Kanyand et al. 1997).

The results described here show the need of exogenous cytokinin for shoot regeneration. Since competence can be regarded as an ability of the explant to respond to inductive stimuli (Christianson and Warnick 1983, 1985) the rapid onset of determination as early as after one-day exposure to BAP suggests that the explant used could be already competent to differentiate shoots before explanting. Similar short time required on the inductive medium was also reported in culture of embryogenic explants of *Picea abies* (von Arnold and Erickson 1985), *Pinus strobus* (Flinn et al. 1988, 1989), *P. ponderosa* (Ellis and Bilderback 1989) and recently zygotic embryos of *T. michelianum* (Konieczny 1996b). In contrast, when more mature explants were used (Biondi and Thorpe 1982, Christianson and Warnick 1983, 1985; Auer et al. 1992) the time of pretreatment on the inductive medium was much more longer and shoot formation was usually preceded by callus production.

In culture of balanse clover the progress in caulogenic determination was accompanied by a decrease in ability of the explant to produce roots, so that at the time of maximum shoot determination rhizogenesis was completely inhibited. Similar relations were also reported in *Medicago sativa* (Walker et al. 1979), *Convolvulus arvensis* (Christianson and Warnick 1983, 1985) and more recently in *Nicotiana tabacum* (Attfield and Evans 1991a, b). According to the Christianson and Warnick (1983, 1985) determination once begun may not be altered or reversed. Thus, at the time of explanting the petioles of balanse clover could be competent for rhizogenesis but not determined. Furthermore, based on the data obtained here it is evident that the induction of shoots and roots required different conditions and was strongly controlled by the growth regulator added to the medium, whereas the requirements for differentiation of an appropriate organ were the same. One of the questions which arose after this experiment is whether the shoots were of the same origin as roots or they originated from different cells. The answer to this question would be very helpful to understand the nature of the competence and state of determination, however it requires advanced cytological and histological studies. Histological observations on shoot versus root formation in balanse clover are in progress.

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ORGANOGENEZA PODCZAS REGENERACJI ROŚLIN
TRIFOLIUM MICHELIANUM SAVI. W KULTURACH IN VITRO LIŚCIENI ZARODKÓW

STRESZCZENIE

Pędy przybyszowe *Trifolium michelianum* Savi. otrzymano z ogonków liścieniowych pochodzących z zarodków hodowanych na pożywce EC6 (Maheswaran i Williams 1984) uzupełnionej BAP w stężeniu 4.4, 8.8 i 22.2 μM . W zależności od ilości użytej cytokininy obserwowano regenerację bezpośrednią lub z kalusa indukowanego z ogonków liścieniowych. Większość pędów po przeniesieniu na MS (Murashige i Skoog, 1962) ze zredukowaną ilością makro- i mikroelementów ukorzeniła się tworząc kwitnące rośliny. Czas indukcji determinacji w kierunku regeneracji pędów wyznaczono na pierwszy dzień kultury. Równolegle z indukcją determinacji do regeneracji pędów obserwowano stopniowy zanik zdolności do ryzogenezy. Obserwacje w SEM pozwoliły na dokładne określenie miejsca indukcji regeneracji pędów: blisko powierzchni odcięcia eksplantatów, z doosiowej i bocznych powierzchni ogonków. Badania w SEM nie wykazały istnienia na powierzchni ogonków liścieniowych miejsc uprzywilejowanych do regeneracji pędów.

SŁOWA KLUCZOWE: determinacja, Leguminosae, organogeneza, regeneracja roślin, *Trifolium*.