HISTOLOGICAL STUDIES ON CALLUS AND SHOOT INDUCTION IN CULTURE OF *TRIFOLIUM NIGRESCENS* VIV. IN VITRO

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The development of callus and adventitious shoots from hypocotyl and cotyledon of *Trifolium nigrescens* seedlings was studied by light microscopy. Calli were formed by multiplication of all living cells of explants except for cotyledonary epidermis within the first week of culture on MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mgl^{-1} NAA and 2 mgl^{-1} 2iP. All the shoots induced from cotyledon- and hypocotyl-derived calli were of multicellular origin and resulted from meristematic cells at peripheral regions of the callus. Copious changes in starch content accompanying callus formation and shoot initiation indicate its significant role in organogenesis of *T. nigrescens*.

Key words: *Trifolium nigrescens*, Leguminosae, organogenesis, histology, callus.

INTRODUCTION

The possibility of plant organ induction under controlled conditions makes tissue culture a unique system for studying plant development. Within the genus *Trifolium*, shoot organogenesis has been successfully induced with a variety of explants as hypocotyls (Mokhtarzadeh and Constatntin, 1978), cotyledons (Phillips and Collins, 1979), leaves (Oelck and Scheider, 1983), flower heads (Skucińska and Miszke, 1980) and zygotic embryos (Beattie and Garrett, 1995). As a result, the major chemical determinants of clover organogenesis in various experimental systems has become well known. However, only a few of the *Trifolium* tissue culture experiments have included histology, so the origins of callus and/or shoot formation in this genus are poorly documented. So far the anatomical changes associated with organogenesis in clover have been reported only in *T. pratense* (Cebrat et al., 1990a,b), *T. repens* (White and Voisey, 1994) and *T. michelianum* (Konieczny, 1996). Knowledge of the developmental events occurring during organ regeneration aids understanding of the general behavior of particular plant species in vitro.

Trifolium nigrescens Viv. (ball clover) is a wild clover species of little agronomic importance (Taylor, 1985). Previously it was found that hypcootyl and cotyledon derived from one-week-old seedlings of this plant can produce organogenic and embryogenic callus, depending on the media composition (Konieczny, 1995). Since the best results for shoot induction were obtained when the explants were cultured on MS (Murashige and Skoog, 1962) containing 0.5 mg l^{-1}NAA and 2 mg l^{-1} 2iP, this medium was chosen for the present studies.

This paper presents a histological characterization of organogenic callus induction and subsequent shoot initiation culture of *T. nigrescens* in vitro.

MATERIALS AND METHODS

Seeds of *Trifolium nigrescens* subsp. *nigrescens* Viv. were provided by the Institut für Pflanzengentik und Kulturpflanzenforschung, Gatersleben, Germany. The seeds were scarified with sandpaper, washed in 70% (v/v) ethanol for 1 min, surface sterilized in 3% (w/v) NaClO for 15 min and then rinsed

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three times in sterile distilled water. Disinfected seeds were germinated on 0.7% (w/v) agar (BBL) solidified MS (Murashige and Skoog, 1962) medium free of growth regulators. After 7 days, hypocotyl explants ~ 0.5 cm in length, as well as the cotyledons, were dissected from seedlings and transferred to MS medium containing 0.5 mgl^{-1} NAA, 2mgl^{-1} 2iP and 0.8% (w/v) agar (BBL). The cotyledons were placed both adaxial and abaxial side down on the medium. The pH of the medium was adjusted to 5.7 with 1N NaOH or 1N HCl before autoclaving. The explants were cultured on 10 cm diameter petri dishes at 25°C with a 16 h photoperiod under cool white fluorescent light at intensity ~100 μM photons $m^{-2}s^{-1}$.

Material for histological analysis was collected daily from day 1 to day 18 of culture. Each probe consisted of 10–15 cotyledons and hypocotyls or calluses. The specimens were fixed with FAA (formalin, acetic acid, 50% ethanol, 5:5:9 v/v/v) for 72 h, dehydrated in a graded ethanol series and embedded in paraffin. Sections cut 7–10 μm thick were stained with acetic carmine and fast green combination, Haidenhain's hematoxylin and periodic acid-Schiff's reagent (PAS) (Jensen, 1962) to reveal starch. The latter treatment was also counterstained with Haidenhain's hematoxylin.

Hypocotyls and cotyledons from embryos germinated on MS free of growth regulators were the control.

RESULTS AND DISCUSSION

HYPOCOTYL CULTURE

Before transfer to culture medium, hypocotyl tissue slices were observed to have a single-layer epidermis, 5–6 layers of cortical parenchyma, and a vascular cylinder of triarch structure (Fig. 1a). The outer layer of the vascular cylinder was a well defined unilayered pericycle consisting of relatively large cells. At this stage there were single starch granules in some inner cortex cells (Fig. 1b; Tab. 1). No divided cells were seen within the hypocotyl.

The first step of morphogenesis in ball clover hypocotyl was starch accumulation in the cortex and epidermis (Fig. 1c; Tab. 1). A similar rapid increase of starch content in explanted potato cotyledons was regarded as the response of the explant to stress associated with in vitro culture initiation (Branca et al., 1994). Gram et al. (1996) reported in culture of pea that accumulation of starch to a certain level could be a prerequisite for cells' ability to enter

TABLE. 1. Starch content in hypocotyl and cotyledon tissues and callus of *Trifolium nigrescens* cultured on MS medium supplemented with 0.5 mg l^{-1}NAA and 2mg l^{-1} 2iP1

Explant	Day of culture				
	0	1	5	10	15
Hypocotyl					
Epidermis	no starch	+	+++		
Cortex	+	++	+++		
Callus				+++	++
Cotyledon					
Epidermis	+	+	+		
Mesophyll	++	++	+++		
Callus				+++	++

Number of crosses corresponds to the number of starch grains: + – single starch grains; +++ – numerous starch grains.

mitosis. Indeed, in ball clover hypocotyls the first cell divisions leading to callus occurred as soon as after 2 days of culture, just after copious starch accumulation in the cortical and epidermal cells. As in red clover (Cebrat et al., 1990a), in culture of *T. nigrescens* the pericycle was found to be the first site of growth regulator action. The rapid activation of the pericycle as the first tissue within the hypocotyl could be due to its meristematic character, among the more differentiated cells of the explant. The divisions of the pericycle in ball clover hypocotyl were regularly periclinal and produced rows of cells of markedly meristematic appearance, rectangular and with a large, centrally positioned nucleus (Fig. 1d). After 1–2 days of culture, mitotic activity extended to the external phloem and inner layers of cortex, leading to the formation of numerous small, darkly staining cells in the central part of the explant (Fig. 1e). After 5–6 days of culture, abundant cell divisions in the outer cortex and some epidermal cells were observed (Fig. 1f). The epidermal cells divided mainly periclinally, but single anticlinal and oblique divisions were also seen. In places where epidermis did not start to divide, internal expansion of the hypocotyl led to the disruption of the epidermis and outgrowth of callus on the surface of the explant. Thus, the hypocotyl-derived callus was formed by the multiplication of all living cells within the explant. The described sequence of events associated with callus formation in ball clover hypocotyls was similar in all explants studied. It closely resembled the data obtained by Cui et al. (1988) in culture of *T. rubens*, and by Reynolds (1989) in *Solanum carolinense*. In contrast, Cebrat et al. (1990a) reported that callus was formed in red clover only by divisions of pericycle cells.

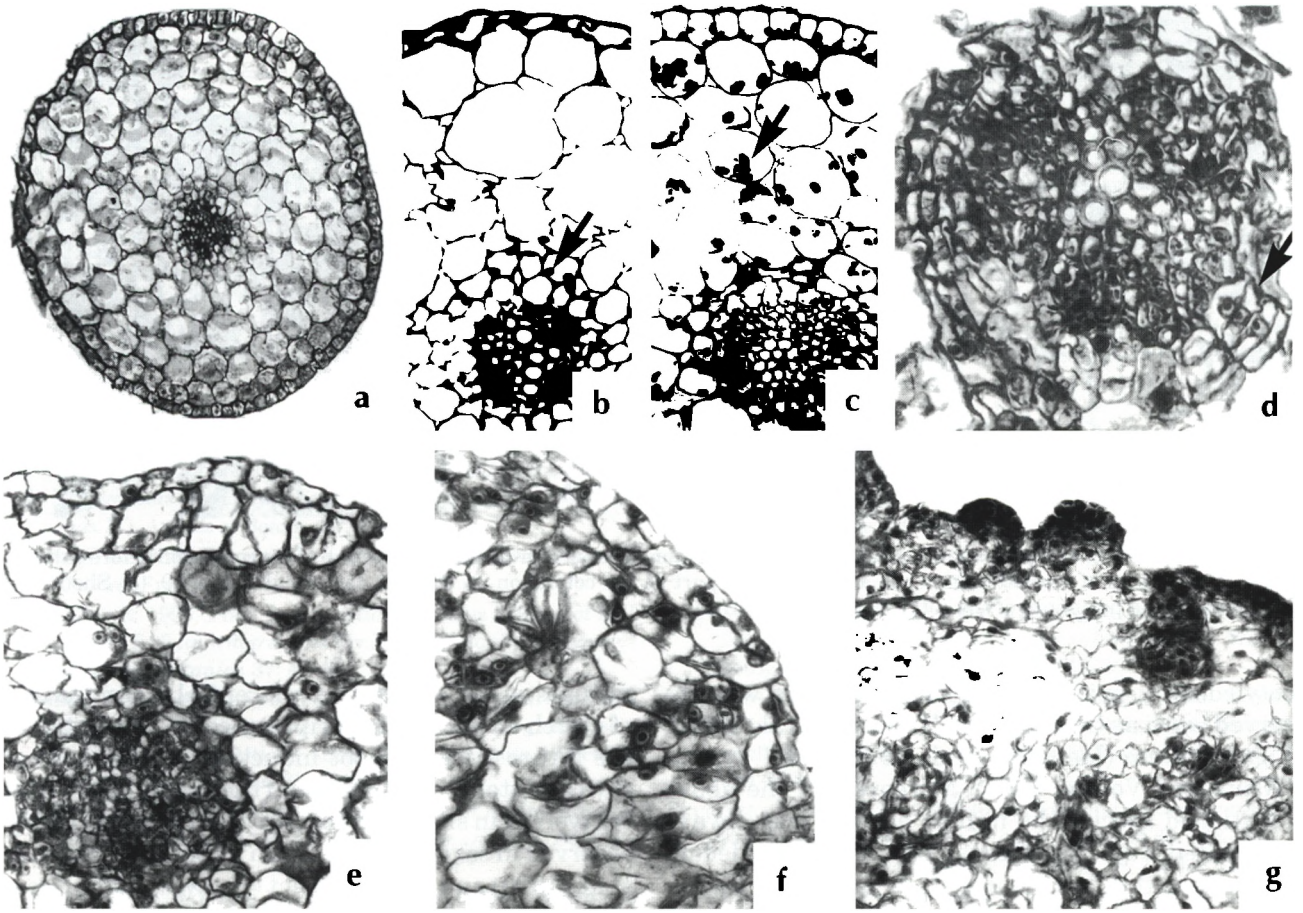

Fig. 1a–g. *Trifolium nigrescens* Viv. Callus and shoot formation in hypocotyl culture. (**a**) Section of the hypocotyl before explanting. × 80, (**b**) Starch content in the hypocotyl before explanting (arrow indicates starch granules). × 150, (**c**) Starch accumulation in hypocotyl after 1 day of culture (arrow indicates starch granules). × 150, (**d**) Induction of cell divisions in the pericycle on the 2nd day of culture (arrow indicates pericyclic derivatives). × 260, (**e**) Induction of cell divisions in the vascular cylinder and cortex on the 4th day of culture. × 130, (**f**) Intense cell division in the cortex and epidermis after 6 days of culture. × 175, (**g**) Organization of shoot meristems at the callus periphery after 10 days of culture. × 100.

The newly formed hypocotyl-derived callus of *T. nigrescens* was homogenous in structure and consisted of rapidly dividing cells without noticeable sites of differentiation. However, after 8–10 days of culture the callus became polarized, having inner regions consisting of large parenchymatous cells with single starch bodies and peripherally arranged meristematic tissue rich in starch. Then, after 14 days of culture, shoot meristems developed from the meristematic poles at the callus periphery (Fig. 1g). As they grew, starch was rapidly depleted in the whole callus tissue (Tab. 1).

COTYLEDON CULTURE

Before explanting, the cotyledon sections showed a single-layer epidermis covering tightly packed, starch-rich mesophyll cells (Fig. 2a). In contrast, single starch granules were observed in epidermal cells (Tab. 1). The central part of the cotyledon was occupied by the vascular bundle with well differentiated xylem and phloem elements. In the control sections of cotyledon no dividing cells were observed.

The first step for morphogenesis in cotyledon culture was the appearance of mitotic activity in the phloem and mesophyll cells close to the vascular bundle (Fig. 2b). The observed cell divisions were oriented anticlinally, periclinally and also in intermediate directions, giving rise to small, cytoplasm-rich cells in the central part of the explant. During the next 2–4 days, mitotic activity was prominent in all the different tissue types of the cotyledon, except for the epidermis. This observation differs from numerous studies in which the involvement of the

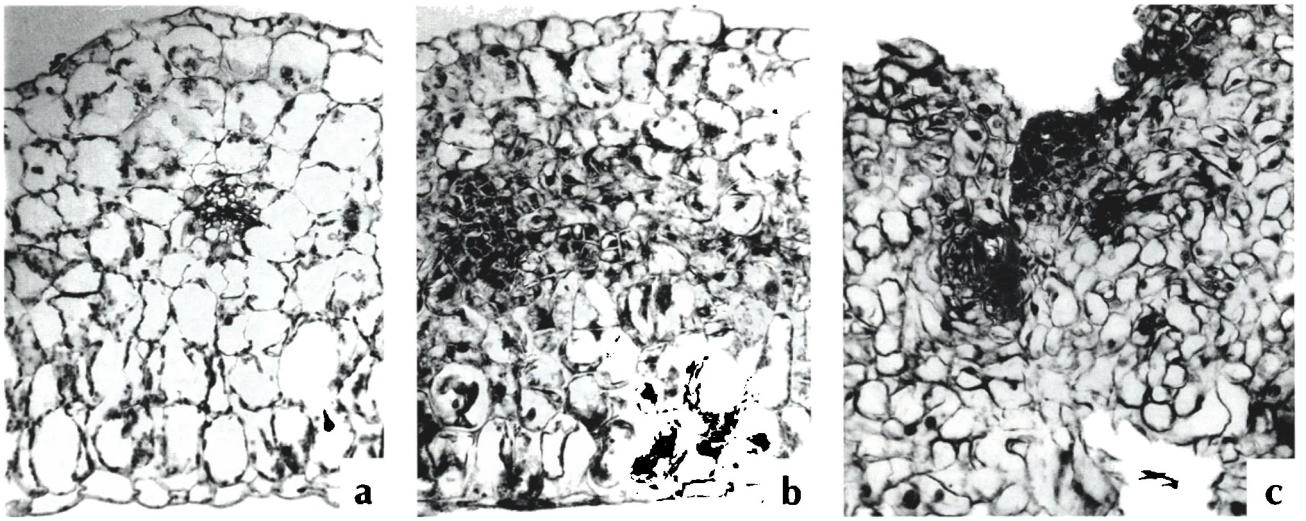

Fig. 2a–c. *Trifolium nigrescens* Viv. Callus and shoot induction in cotyledon culture. (**a**) Section of the cotyledon before culture. × 70, (**b**) Clusters of meristematic cells at the central part of the cotyledon on the 8th day of culture. × 70, (**c**) Shoot bud initiation at the periphery of the cotyledon callus. × 110.

cotyledonary epidermis in morphogenesis was observed, such as *Glycine max* (Hartweck et al., 1988), *Vigna radiata* (Mendoza et al., 1993) and *T. repens* (White and Voisey, 1994). Moreover, in the above mentioned species the mitotic activity was always associated with the adaxial side of the cotyledon, whereas within the mesophyll of ball clover cotyledons no preferential sites of cell multiplication were observed. As a result, at the beginning of the second week of culture the callus ruptured both the abaxial and adaxial epidermis. As described previously for soybean (Hepher et al., 1988), the pattern of callus development in ball clover cotyledons was independent of the mode of explant culture on the medium (adaxial or abaxial side down). In 10-day-old cotyledonary callus the mitotic activity occurred preferentially in certain zones at the callus periphery. After 14–16 days of culture, nodule-like structures and finally shoot meristems arose from these zones (Fig. 2c).

The data from this study make it apparent that the shoots induced from cotyledon- and hypocotyl-derived calli of ball clover were formed by the multiplication of several cells at the callus periphery. The exogenous origin of shoots in callus culture was recently reported in *T. michelianum* (Konieczny, 1996) and *Papaver somniferum* (Ovecka et al., 1997). Cebrat et al. (1990a), however, found that in red clover the shoots regularly were formed endogenously, deep in the callus tissue. The reasons for the differences in the location of shoot induction sites within the calli are difficult to explain. However, in calluses of ball clover the preferential accumulation of starch at the callus periphery could be related to the future sites of shoot induction. An increase of starch content in the regions of the explant ultimately involved in shoot bud regeneration was previously observed in several species such as *Begonia* (Magnat et al., 1990) and African violet (Redway, 1991). Despite the clear correlation between starch content and the course of organogenesis in ball clover (Tab. 1), its role in the regulation of shoot formation processes in this plant requires further studies.

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