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SOMATIC EMBRYOGENESIS FROM BROCCOLI STIGMAS IN TISSUE CULTURE

MACIEJ ZENKTELER*, ELŻBIETA ZENKTELER, AND IWONA DOSTATNIA

Department of General Botany, Institute of Experimental Biology, Adam Mickiewicz University, ul. Umultowska 89, 61-614 Poznań, Poland

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The factors regulating callus proliferation and bud regeneration from stigma tissues are not sufficiently understood. To study the regenerative capacity of pistil elements, pistil of broccoli was cultured under simple culture conditions. Stigmas with style from broccoli pistils undergo somatic embryogenesis on Murashige and Skoog basal medium. Callus initiation occurred on basal medium supplemented with 4 $mg \cdot l^{-1}$ BAP, 1.6 $mg \cdot l^{-1}$ 2,4-D, 250 $mg \cdot l^{-1}$ casein hydrolysate and 30 $g \cdot l^{-1}$ sucrose. Proembryo induction was observed after two callus subcultures. Calluses with globular embryos were cultured on basal medium with 2 $mg \cdot l^{-1}$ BAP, 1 $mg \cdot l^{-1}$ IAA and 40 $g \cdot l^{-1}$ sucrose for development, maturation and germination of somatic embryos. A population of somatic embryos was maintained on medium containing 1 $mg \cdot l^{-1}$ BAP and 2 $mg \cdot l^{-1}$ NAA only. Adding NAA to MS medium containing BAP considerably enhanced root formation. After acclimatization, all plantlets developed well and produced phenotypically normal flowers.

Key words: *Brassica oleracea* L. var. *italica* subvar. Cymosa, plant regeneration, stigma culture, somatic embryogenesis.

INTRODUCTION

Many types of proteins are involved in pollen-pistil interaction in the secretory and parenchymatous cells of the stigma and the transmitting cells of the style. These proteins include self-incompatibility-related S-RNAses in the family Solanaceae (Cornish et al., 1987) and transmitting-tissue-specific glycoproteins attracting pollen tubes in *Nicotiana* (Cheung et al., 1996). PR-like proteins are abundant in the stylar transmitting tissue; they act to protect the stigma against potential pathogens (Kuboyama, 1998). Thus, stigmatic and stylar tissues play very important roles in reproductive processes (Labarca and Loewus, 1973). Their morphogenetic potential in vitro has not been investigated, however. This study was intended to determine whether secretory tissues of the broccoli stigma, such as papillae and transmitting tissue, have the capacity to proliferate callus, and whether whole plants can regenerate from the callus.

MATERIALS AND METHODS

Inflorescences of broccoli *Brassica oleracea* L. var. *italica* subvar. Cymosa were collected from an experimental plantation of the Agricultural Academy of Poznań. Closed flower buds of broccoli (with petals protruding only 1–2 mm beyond the sepals) were isolated from the inflorescences, then soaked in 70% (v/v) ethanol for 5 sec, disinfected with 2% (v/v) NaOCl + Tween 80 for 6 min, followed by three rinses in sterile distilled water. Young stigmas with or without style were placed vertically or horizontally on MS medium (Murashige and Skoog, 1962). Half the explants were additionally scarified by incision or puncture. Twenty explants per treatment were subcultured in Petri dishes (6 cm diam) containing 20 ml medium and covered with parafilm. Each treatment was done in triplicate. Subcultures were performed at 4-week intervals. Ten variants of MS medium were used for propagation in the study (Tab. 2). The pH of the medium was adjusted to 5.6 before autoclaving at 105 kPa for 10 min. Explants were incubated at 21±2°C under continuous cool white fluorescent light (250 $\mu mol\ m^{-2}\ s^{-1}$) at 80% RH in a growth chamber. Regeneration percentage was calculated.

Material for anatomical study was collected 0, 7, 15 and 30 days after isolation and transfer of calluses to the modified MS medium. Samples were fixed in FAA (formalin, acetic acid, ethanol, water

*e-mail: maczen@amu.edu.pl

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TABLE 1. Effect of mechanical stress and position of explants on callus proliferation from stigma of broccoli with and without style on MS medium

Treatment	Stigma with style		Isolated stigma	
Position	Vertical	Horizontal	Vertical	Horizontal
Stigma not scarified	15.1%	21.1%	7.3%	8.8%
Stigma scarified	18.8%	31.7%	11.6%	15.6%
Stigma punctured	20.4%	28.7%	10.5%	16.2%

10/5/50/35) for 24 h, dehydrated in an ethanol-xylene series and embedded in paraffin. Thin sections (12 μm) were double-stained with 1% (w/v) safranin and 1% (w/v) fast green. Slides were observed and photographed under a Zeiss Axioscope light microscope.

RESULTS

CHARACTERISTICS OF BROCCOLI STIGMA STRUCTURE

The broccoli pistil is composed of the stigma, short style and ovary. In the upper part of the pistil, stigmatic secretory tissue is connected to stylar transmitting tissue extending along the axis of the style to the top of the ovary and merging with the lining of the ovary. Morphological analysis of the stigma's receptive surface revealed the broccoli stigma to be of dry type, covered with a layer of unicellular papillae (group II according to the classification of Heslop-Harrison, 1981). The exudate secreted by the papillae is protected by a pellicle and a layer of wax. The stigmatic glandular epidermis is limited to a thin layer of highly vacuolated papillae. The unicellular papillae are elongated, and their bases adhere to one another. Their basipetal nuclei are four times larger than the nuclei of the underlying transmitting tissue (Fig. 1). The secretion is produced and accumulated extracellularly between the papillae of the stigma of closed flower buds before anthesis.

The broccoli pistil is a typical closed pistil with well-developed transmitting tissue built of elongated cells (Heslop-Harrison, 1981). It is characterized by a specific arrangement of cells: vertical in the central part of the stigma and in the style, and horizontal at the edges of the stigma. Two protuberances with a smooth surface are visible at the top of the stigma. The protuberances are filled with irregular parenchymatous cells surrounding the endings of two vascular bundles (Fig. 1).

EFFECTS OF EXPLANT ORIENTATION AND MECHANICAL STRESS ON CALLUS PROLIFERATION

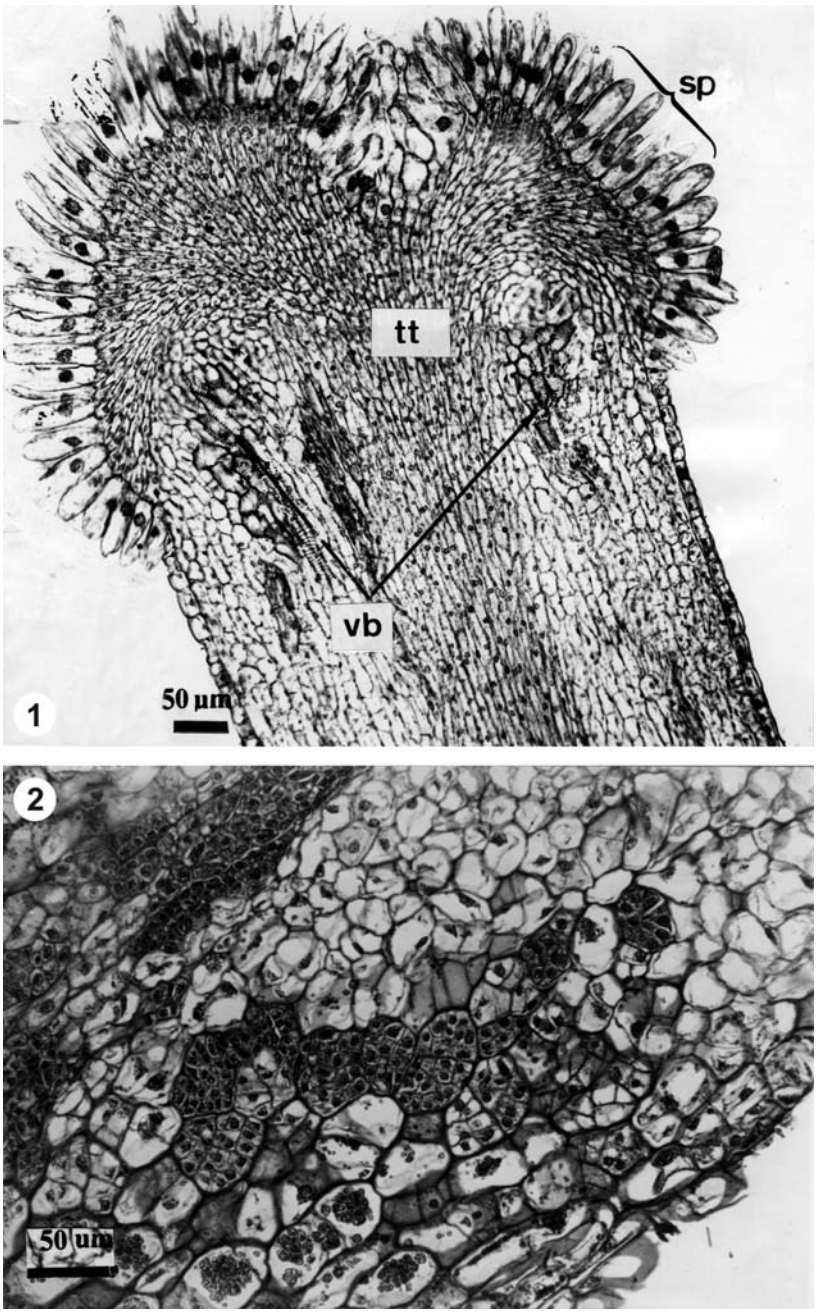
The largest amounts of callus were produced by horizontally oriented stigmas with a style (Tab. 1, Fig. 3). In this combination the percentage of callusing explants was nearly twice that of vertically oriented explants. Stigmas isolated from the style proliferated less intensively than stigmas with a style. The stress caused by incision or puncture increased the number of callusing explants. Puncture proved to be a stronger inducing factor than incision (Tab. 1).

Histological examination confirmed that callus regenerated better from stigmatic tissues connected to a style than from isolated stigmas. Since much of our work was concerned with the histology of callus formation, it is still unclear whether only parenchyma tissue initiated callus proliferation. However, examination of all sections revealed that callus did not originate from papillae or stylar epidermis.

TABLE 2. Effect of growth regulators (in $mg \cdot l^{-1}$) in MS modified media on callus proliferation from stigmas with a style; somatic embryogenesis and rooting of plantlets of broccoli

Callus proliferation on initiation medium			Embryo development on regeneration medium			Plantlet rhizogenesis on rooting medium		
BAP	2,4-D	%	BAP	IAA	%	BAP	NAA	%
2	0.6	15.3	2	1	26.6	0.5	1	31.1
4	1.6	21.7	4	2	31.4	1	2	38.5
6	2	10.8	6	3	22.9	1.5	3	24.7
control *		0.9	control		7.5	control		12.6

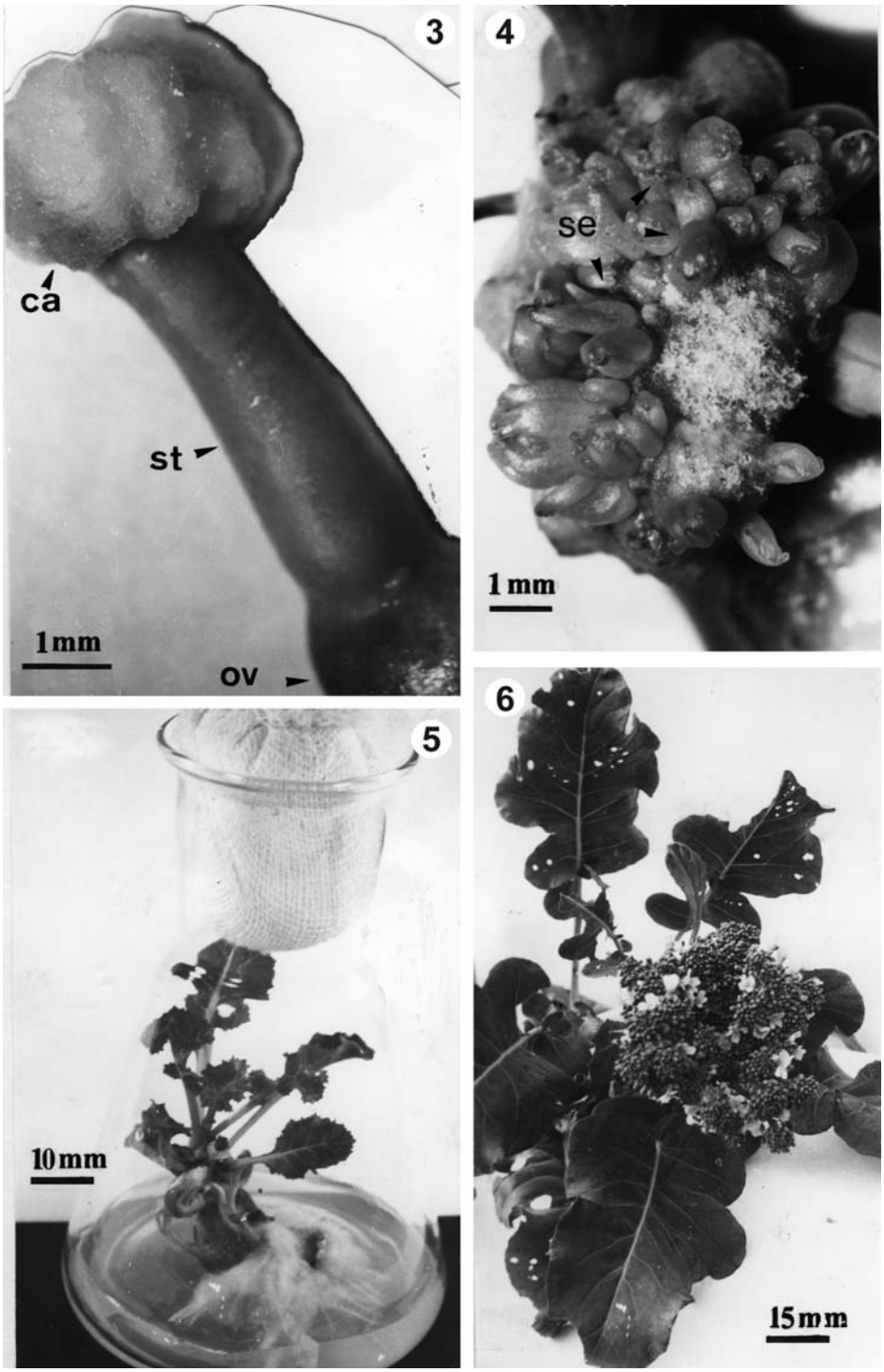
* medium without growth regulators



Figs. 1, 2. Longitudinal section through stigma, style and callus derived from explants of broccoli. **Fig. 1**. Median longitudinal section of stigma and upper part of style before in vitro culture. sp – secretory papillae; tt – transmitting tissue; vb – vascular bundle. **Fig. 2**. Globular embryos developing from callus after 15 days of culture on MS medium supplemented with 4 $mg \cdot l^{-1}$ BAP, 1.6 $mg \cdot l^{-1}$ 2,4-D, 30 $g \cdot l^{-1}$ casein hydrolysate and 30 $g \cdot l^{-1}$ sucrose.

EFFECTS OF GROWTH REGULATORS ON CALLUS PROLIFERATION AND EMBRYOGENESIS

Three optimum variants of MS medium were selected for (i) inducing callus proliferation, (ii) inducing proembryos, and (iii) converting proembryos into cotyledonary embryos. The optimum callus proliferation, from 21.1% of explants (Tab. 2), was achieved on MS medium supplemented with 4 $mg \cdot l^{-1}$ BAP, 1.6 $mg \cdot l^{-1}$ 2,4-D, 250 $mg \cdot l^{-1}$ casein hydrolysate and 30 $g \cdot l^{-1}$ sucrose (Fig. 3). The stigmas expanded as early as 7–10 days after placing explants on the inducing medium, and were later covered by proliferating callus. The callus was compact, hard white/cream and contained regions quickly turning



Figs. 3-6. Induction of callus, embryo and plant regeneration from stigma of *Brassica oleracea* L. var. *italica* subvar. Cymosa. **Fig. 3**. Callus developing from scarified stigma with style placed vertically on MS medium supplemented with 4 mg·l^{-1} BAP, 1.6 mg·l^{-1} 2,4-D, 30 g·l^{-1} casein hydrolysate and 30 g·l^{-1} sucrose. Three weeks after explant isolation. ca – callus; st – style; ov – part of ovary. **Fig. 4**. Numerous mature cotyledonary embryos (se) protruding on surface of callus on MS medium supplemented with 2 mg·l^{-1} BAP, 1 mg·l^{-1} IAA and 40 g·l^{-1} sucrose after 35 days of culture. **Fig. 5**. Rooting of plantlets obtained from embryos. Roots were induced on MS medium supplemented with 1 mg ·l^{-1} BAP and 2 mg·l^{-1} NAA. Bar = 50 μm. **Fig. 6**. Flowering broccoli plant regenerated via somatic embryogenesis from stigmatic explant, four months after transferring to soil.

green. At 10 days after explant isolation, only parenchymatous cells were found in white regions of callus. Many proembryos could be observed just below the surface in green areas of callus (Fig. 2). Starch grains of various sizes were found in the cells surrounding these regions.

On day 35 the proliferating callus was separated from the initial explants and transferred to MS medium containing 2 $mg \cdot l^{-1}$ BAP, 1 $mg \cdot l^{-1}$ IAA and 40 $g \cdot l^{-1}$ sucrose (Tab. 2). On this medium, white cotyledonary embryos, clearly polarized, protruded at the surface of callus aggregates in 31.4% of the explants (Fig. 4). When regenerated embryos with two cotyledons and a radicle reached 2–3 cm in length, they were transferred to rooting medium with 1 $mg \cdot l^{-1}$ BAP and 2 $mg \cdot l^{-1}$ NAA (Tab. 2). In that medium, 38.5% of the explants rooted properly (Fig. 5). About four months were needed to obtain rooted broccoli plantlets, which were planted in pots in a greenhouse and soon started flowering (Fig. 6).

DISCUSSION

Pistil maturation in broccoli occurs basipetally, starting from the stigma and proceeding down to the ovary. As the earliest maturing element, the stigma starts to age earlier than the other elements. The period of activity and the viability of transmitting tissue in the stigma is shorter than in the style (Raghavan, 1997). This might have been one reason for poor callus proliferation. Alternatively, poor callusing of isolated stigmas might have been caused by separation from the other parts of the pistil. Stigmas that had not been isolated from the style used nutrients supplied by the transmitting tissue of the style, which secreted the largest amounts of exudates. The secretion in the stylar transmitting tissue contains glucose and galactose, which serve as energy sources for the growing pollen tubes (Bell and Hicks, 1976). Probably because of this, stimulation with growth regulators induced the most intensive proliferation of callus from stigmas connected with the style. Our results also indicate that callus formation depends on the extent of contact of the explants with the growth regulators contained in the medium, as confirmed by the finding of most abundant callus proliferation from incised stigmas oriented horizontally, in direct contact with the medium. Stigmas on pistils oriented vertically produced much less callus.

High concentrations of sucrose in the medium improve development and maturation of somatic embryos of broccoli. Sucrose supplementation also enhances embryo conversion to plantlets in some other plant species (Krishnamurthy, 1999). During conversion of broccoli embryo structures into mature cotyledonary embryos, some disturbances in their development were found. Malformations such as fasciation were also related to the number of embryos in one cluster. After separation, normal embryos appeared clearly polarized, with two cotyledons and a radicle.

This is the first report of induction and conversion of somatic embryos to plants via callus obtained from stigma tissues of broccoli.

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