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Direct somatic embryogenesis and plant regeneration from immature embryos of hybrid sunflower (*Helianthus annuus* L.) on a high sucrose-containing medium*

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ABSTRACT

Somatic embryos of sunflower (*Helianthus annuus*) were obtained by placing immature zygotic embryos on a high sucrose (12%) containing medium. The somatic embryos were first observed 6 days after culture and a callus intermediate was not formed. Histological examination revealed the classical stages of embryo development. The somatic embryos proliferated directly from the surface of the zygotic embryos and germinated after placement on a low sucrose medium. Seeds have been obtained from regenerated plants.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid, NAA = 1-naphthaleneacetic acid, IAA = Indole-3-acetic acid, BAP = 6-benzylamino purine.

INTRODUCTION

Sunflower (*Helianthus annuus*) tissue has typically been very easy to culture. Normal sunflower callus (Hildebrandt and Riker, 1946) and transformed sunflower tissues (DeRopp, 1947) were established *in vitro* in the 1940's but regeneration was not reported. Sunflower was first regenerated by Sadhu (1974) who cultured stem pith on a medium containing 1 mg/l IAA. Differentiation of shoots was observed from a single callus after 10 weeks on this medium. Plant regeneration was reported but the cultivar utilized was not named. In 1981, Binding *et al.* reported regeneration of sunflower from protoplasts. As this was a comprehensive study involving 77 dicotyledonous plants, details of the procedure as well as the cultivar used were not mentioned. Recently, Lenee and Chupeau (1986) and Bohorova *et al.* (1986) reported protoplast isolation and culture of cultivated sunflower (*Helianthus annuus* L.). In both reports, callus was regenerated from protoplasts but plantlets were not obtained from that callus. However, information was presented (as unpublished data) on plant regeneration from protoplasts of a wild sunflower species (Bohorova *et al.*, 1986).

Greco *et al.* (1984) and Paterson and Everett (1985) provided the first detailed reports of regeneration of sunflower. Using seedling tissue from the synthetic variety "Sannace", Greco *et al.* (1984) obtained shoot regeneration if BAP was the sole growth regulator present in the medium. Shoots were obtained after one month in culture and a callus intermediate was formed. Paterson and Everett (1985) also utilized seedling tissue but from the economically important inbred SS415B. Seedling age as well as additional KNO_3 were important in optimizing regeneration. Green spots, which formed in a callus after 3 weeks in culture, gave rise to shoots. The shoots were then rooted to form plants. Through histological examination, the authors reported that the green spots were somatic embryos.

In this report, immature hybrid zygotic embryos were used for initiation of somatic embryogenesis. Somatic embryos arose directly from the cotyledons of the zygotic embryo without the formation of a callus intermediate. Embryos were initially observed as early as 6 days after culture.

MATERIALS AND METHODS

Sunflower inbred and hybrid seeds were obtained from Mary Lou Straley (Cargill Seeds, Fargo, ND). Inbred HA401 (cytoplasmic male sterile) and HA699 (restorer) plants were grown in greenhouses at Wooster, OH with natural lighting from April to October and supplemental lighting from November to March. After flowering, pollinations were performed daily for 1 week. The HA401 X HA699 cross gave rise to hybrid immature embryos of C204. These embryos were utilized as explants from 7 to 14 days post-pollination. Intact immature seeds as well as excised mature zygotic embryos of hybrid C204 were surface sterilized by placement in a 20% commercial bleach solution containing 0.05% Tween-20. After 20 min, the embryos and seeds were rinsed 4 times with sterile, distilled water.

Excised mature zygotic embryos were then placed on a hormone-free medium (OMS) containing Murashige and Skoog's (MS) salts (1962), Gamborg's B-5 vitamins (1968), 2% sucrose and solidified with 0.8% agar (pH 5.7). The mature zygotic embryos and the seedling tissue derived from the germinating embryos (1-7 days post-planting) were placed on a medium containing MS salts, B-5 vitamins, 12% sucrose, 1 mg/l 2,4-D, and solidified with 0.8% agar (pH 5.7). All cultures were maintained at 28°C with a light intensity of 25 $\mu Em^{-2}s^{-1}$ and a 16:8 hour light:dark photoperiod.

Immature zygotic embryos were excised from the seed and placed on various media. These media contained MS salts, B-5 vitamins and varying levels of sucrose, 2,4-D, dicamba (Velsicol; Chicago, IL), picloram (Dow; Midland, MI), NAA and IAA. Media were evaluated based on frequency of initiation of somatic embryos after 1, 2 and 4 weeks in culture.

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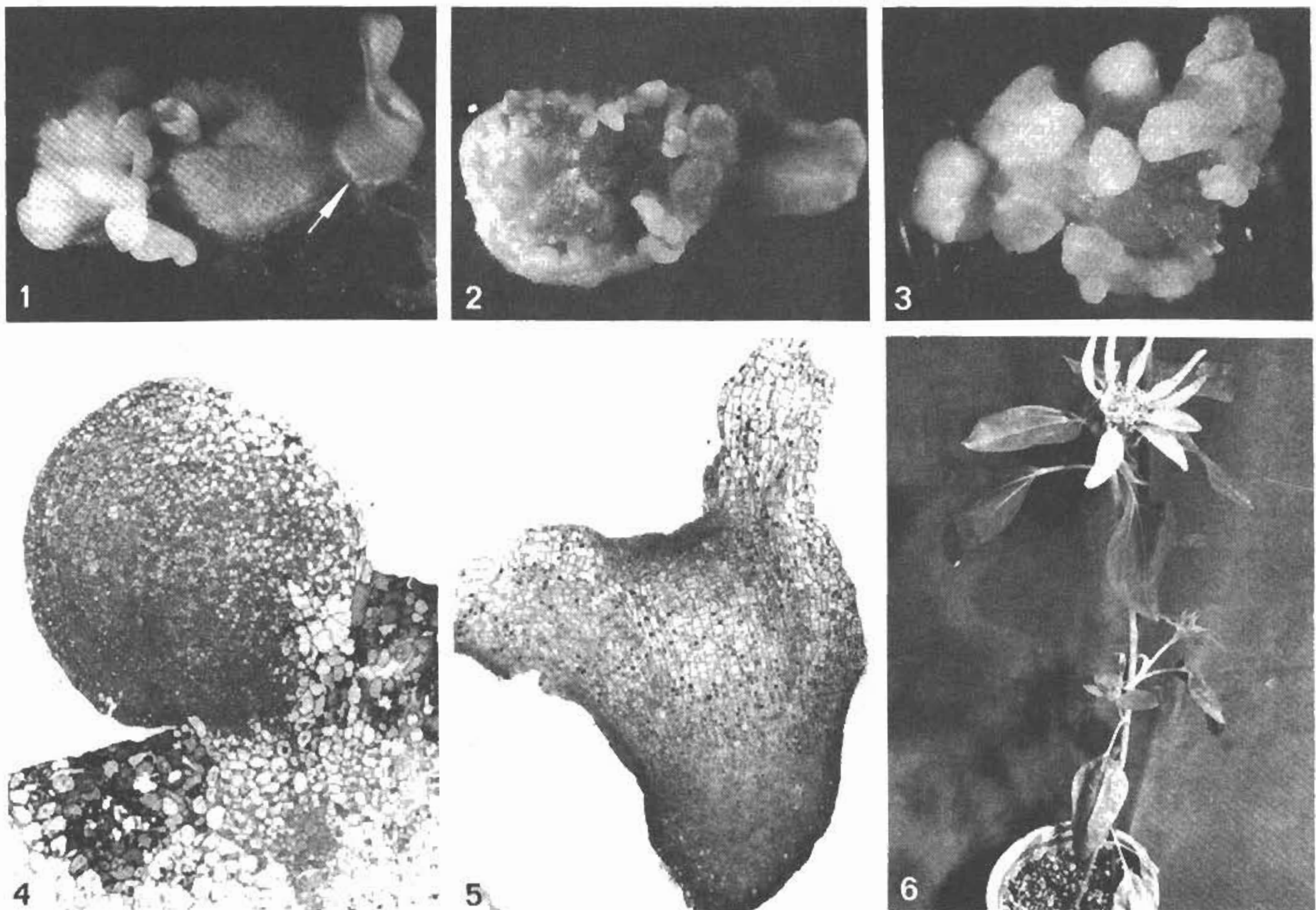
Following initiation of somatic embryogenesis, the somatic embryos were either placed on the OMS medium for embryo development and germination or subcultured onto auxin-containing medium for continued proliferation of somatic embryos. Plantlets derived from somatic embryos were transferred to vermiculite and watered with 1/4 strength MS salts. Plantlets were initially covered and gradually exposed to ambient humidity over a 2 week period.

For histological studies, primary somatic embryos were removed from the cotyledonary surface and fixed in a 0.2 M phosphate buffer solution (pH 7.4) containing 3% glutaraldehyde, 2% paraformaldehyde, and 1.5% acrolein. Following an overnight fixation at room temperature, the embryos were rinsed 3 times in 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). The tissue was then rinsed with cold water, stained overnight in 1% uranyl acetate, and dehydrated in an ethanol series. Somatic embryos were embedded in Spurr's resin (1969) and cut to 0.75 μm on a JB-4 microtome. Sections were affixed to glass slides by heating and stained with toluidine blue for viewing.

RESULTS AND DISCUSSION

Somatic embryos were first observed 6 days after culture. In contrast to the "green spots" reported by Paterson and Everett (1985), the somatic embryos described in this report were initially white and proliferated directly from the surface of the cotyledons of the immature zygotic embryos. Although some somatic embryos were produced on the middle and terminal portions of the cotyledons (Figure 1), the majority of the embryos were formed on the edge and basal portions (Figure 2). In rare cases, the zygotic embryo became covered with somatic embryos (Figure 3).

No somatic embryos were formed when 3% sucrose was used in combination with IAA, NAA, picloram, dicamba, or 2,4-D at 0.1, 0.5, 1, 5, or 10 mg/l. When 6% and 12% sucrose were used in combination with 2,4-D at 0.3, 1, and 3.3 mg/l, or dicamba at 3.3, 10, and 33 mg/l, somatic embryos were formed (Table I). Dicamba at 3.3 mg/l and 2,4-D at 1 mg/l were optimum for somatic embryo initiation from zygotic embryos. No somatic embryos were formed when mature embryos or seedling tissues were placed on a medium which induced somatic embryogenesis from



Figures 1-6. Regeneration through somatic embryogenesis of sunflower. Fig 1. Somatic embryos of sunflower formed on terminal portion of cotyledon. Arrow indicates zygotic embryo shoot tip (X 4.3). Fig. 2. Sunflower zygotic embryo forming somatic embryos from cotyledon basal tissues (X 4.3). Fig. 3. Somatic embryos covering surface of zygotic embryo (X 4.1). Fig. 4. Section of globular somatic embryo (X 145). Fig. 5. Section of torpedo-shaped embryo (X 66). Fig. 6. Regenerated plant (X 0.2).

Table I Effect of sucrose concentration and auxin level on percent zygotic embryos forming somatic embryos.

Auxin	% zygotic embryos forming somatic embryos ± S.E.	
	6% sucrose	12% sucrose
3.3 mg/l dicamba	3.8 ± 3.4	21.5 ± 10.1
10 mg/l dicamba	12.5 ± 6.3	8.3 ± 14.4
33 mg/l dicamba	16.7 ± 14.4	12.5 ± 16.5
0.33 mg/l 2,4-D	8.3 ± 7.2	15.0 ± 18.0
1 mg/l 2,4-D	10.4 ± 9.5	31.2 ± 11.7
3.3 mg/l 2,4-D	0	6.3 ± 4.4

immature zygotic embryos. Chandler and Beard (1983) and Espinasse *et al.* (1986) used a high sucrose medium to allow maturation of immature sunflower hybrid zygotic embryos. After these embryos developed on the high sucrose medium, they were transferred to a low sucrose medium for germination. A system reported by Wilcox (1986) appears similar to the system in this paper. In that system, immature embryos from 7 different inbred lines of sunflower were plated on a medium containing 12% sucrose. Tissue, which formed on the cotyledons, gave rise to shoots when transferred to a low sucrose medium. Further comparison is difficult as details of the system were not given (Wilcox, 1986).

Histological examination of sunflower somatic embryos revealed the classical stages of embryogenesis. The globular staged embryos (Figure 4) were continuous with the cotyledonary epidermis. There was no clear internal organization although an epidermis was present. Sunflower somatic embryos at the torpedo-shaped state (Figure 5) showed much internal organization. Epidermal tissue, cotyledon primordia, vascular traces, and both root and shoot meristems were apparent in these embryos.

As a continual source of flowering plants was necessary to obtain the immature zygotic embryos used for somatic embryo induction, most of the somatic embryos and embryogenic tissue obtained in this report was used for maintenance experiments. Attempts to maintain embryogenic tissue on a 2,4-D-containing medium were unsuccessful. The tissue turned brown after 2 monthly subcultures. Embryogenic tissue could be subcultured and maintained on media containing dicamba at 3.3 and 10 mg/l in combination with 6% or 12% sucrose. Embryogenic tissue has been maintained for 7 months.

After transfer of the mature embryos to the hormone-free OMS medium, 20% of the embryos germinated. Root elongation was apparent in the remainder of the plated embryos. Only 2 germinating embryos gave rise to complete plants (Figure 6). The plantlets flowered early, were smaller than hybrid plants from seed, and were fertile.

Although this is not the first report of regeneration in sunflower, the method of regeneration is different from existing reports. Immature embryos from an economically important hybrid were utilized in this work rather than embryo (Wilcox, 1986), or seedling (Greco *et al.*, 1984; Patterson and Everett, 1985) tissues from inbreds. Histological evidence indicated that somatic embryogenesis and not shoot morphogenesis (Wilcox, 1986; Greco *et al.*, 1984) was the route of regeneration. Somatic embryos were observed as early as 6 days after culture and a callus intermediate was not formed. Plant regeneration has been achieved. This system will be used to study the physiology of embryogenesis using embryo-specific cDNAs obtained from zygotic sunflower embryos (Allen *et al.*, 1985). Attempts are currently underway to further optimize the somatic embryo induction process, regenerate plants from embryogenic tissue maintained in culture for extended periods, and obtain embryogenic suspension cultures.

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