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SHORTENING BREEDING CYCLE THOUGH IMMATURE EMBRYO CULTURE IN SUNFLOWER (*Helianthus annuus* L.).

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SUMMARY

Immature zygotic hybrid embryos of sunflower were cultured on MS medium supplemented with BAP in the range of 0.5 02.5 mg/l. Adventitious shoot buds were induced on the hypocotyl region of the immature embryos of a cross between a commercial male sterile line (234A) and a restorer line (RF1). These adventitious buds were grown to shoots, rooted and transferred to the field. Fertile plants were obtained which set seeds in approximately two and a half months from initiation. This method will help shorten generation time an therefore can be profitably used in sunflower breeding programmes.

Key words: *Helianthus annuus*, embryo culture, crop improvement, mutiple shoots.

INTRODUCTION

Regeneration in sunflower (*Helianthus annuus* L.) through somatic embryos from various cultured explants has been attempted by several group of workers (Nataraja and Ganapathi, 1989; Greco et al., 1984; Sandhu, 1974). Robinson and Everett (1990) have adequately emphasized the wide-ranging efforts on a regeneration system in sunflower and the limited success met with thus far. This recalcitrancy is due to the highly genotype specific behaviour in culture of this important oil seed crop. Nevertheless, regeneration of fertile plants from culture of immature zygotic embryos has been reported (Assad et al., 1986; Finner, 1987; Freyssinet&Freyssinet, 1988; McCann et al., 1988; Wirtzens et al., 1988; Espinasse et al., 1989; and Jeannin and Hahne, 1991). Its importance in the breeding programme has been emphasized by Virupakashappa (1987).

This study was aimed at evaluating the usefulness of immature embryo culture in shortening the breeding cycle of sunflower. Fertile plants were regenerated from the hypocotyl region of immature embryo explants and these set seeds in about two and a half months from culture initiation. This method could be a useful adjuvant to a combination breeding programme.

MATERIALS AND METHODS[1]

Plant material: Immature embryos resulting from a cross between a CMS line (234A) and a restorer line (RF1) were excised from immature seeds harvested 3,5,7,9, and 11 days after pollination for initiating cultures.

Culture conditions: Immature embryos were inoculated in MS (Murashige and Skoog, 1962) based media amended with various growth hormones and other additives as listed in Table 1. The pH of all media was adjusted to 5.8, with 0.1N NaOH and autoclaved at 121°C for 20 min. The media were poured into sterile petri plates and the plates with medium stored for a week at room temperature. Immature seeds were surface sterilized by soaking in 70% ethanol for 30 seconds and then in 10% commercial bleach (calcium hypochlorite) for 15 min. Seed coats were removed and the aseptically isolated embryos were inoculated on the medium with the radicle end pointing downwards. The cultures were kept at 25±1°C with a photoperiod cycle of 16h light (ca. 6000 lux) and 8h darkness.

Table 1: Hormone combinations used for immature embryo culture in MS medium with 9% sucrose.

Medium code	E	E1	ED**	EMS
BAP mg/l	0.5	1.0	1.0	2.5
**Supplemented with 390 mg/l mesoinositol; 10 mg/l L-cysteine HC; 50 mg/l tryptophan; 800 mg/l glutamine; 1.5 g/l casamino acid.				

Table 2: Number of adventitious shoot buds induced per inoculated embryo two weeks after initiation.

Age of embryo	Medium code			
	E	E_1	ED	EMS
3 day	0.0	0.3	0.5	0.0
5 day	1.6	1.2	0.9	0.3
7 day	2.0	0.2	0.3	0.6
9 day	1.3	1.6	0.9	1.1
11 day	0.0	0.1	0.2	0.5
Ten explants per treatment were initiated.				

Table 3: Number of plants obtained from each stage of the zygotic embryo.

		Medium			
Age of embryo	size	E	E_1	ED	EMS
3 day	1mm	0	6	0	0
5 day	3mm	1	6	4	0
7 day	5mm	13	5	4	0
9 day	7mm	13	8	3	5
11 day	8mm	3	0	6	3

1 Abbrevations: BAP- 6-benzyl amino-purine;2,4-D - 2,4-dichlorophenoxy acetic acid; FAA - formaldehyde/acetic acid/ alcohol

The embryos germinated and gave rise to green cotyledon leaves and roots in about seven days. In the green hypocotyl region multiple adventitious buds were seen 10-15 days after initiation in the medium. The multiple shoots along with the hypocotyl were excised and subcultured on the same medium for a futher period of 10-15 days until the buds grew to a height of 2-5cm. They were then individually transferred to MS basal medium with a lower sucrose concentration of 3% (with or without charcoal at 1 g/l) for rooting.

In vitro rooted plants grown to a height of 3-6 cm were potted into autoclaved plastic pots containing 3:2:1 peat, perlite and sand, respectively. These were kept in a growth chamber at 25±1°C and with a photoperiod cycle of 16h light and 8h darkness. The RH was maintained at 90% for the first 3 days and then reduced to 70%.

Histological studies: The vegetative bud along with a portion of hypocotyl was prefixed in FAA (5:5:90) overnight. The specimen was prepared for microscopic examination as described by Johansen (1940).

RESULTS

The response of the immature embryos varied with their age and the culture medium (Table 2). The inoculated embryos germinated in 3-4 days to produce cotyledon leaves and a tap root. In most media combinations adventitious buds were produced from the hypocotyl region in about a week. In two weeks, the adventitious buds were sufficiently grown (Fig.1a); at this stage the main shoot and root were cut off and the buds cultured in the same medium. Of the four media tested, the medium E, which contained 0.5mg/l BAP, was found to be the best for multiple adventitious shoot induction. The 3 day old embryo did not produce any multiple shoots in this medium, even though it produced multiple shoots in media containing higher concentrations of BAP.

The 3 and 5 day old embryos showed multiple shooting response in less than 7 days while the more aged embryos took about 10 days. The older embryos, however, produced more adventitious shoots per inoculated embryo. The response of the eleven day old embryo was very slow; multiple shoots were visible only after about 3 weeks in culture. The seven day old embryos in the medium E showed the best multiple shooting response with an average of 2 adventitious shoots per embryo.

Histological analysis of the adventitious buds showed shoot primordia and separate vasculature for each bud (Figure 1b). The root primordia were absent indicating that these were adventitious shoots and not somatic embryos.

The adventitious buds were transferred to the rooting medium when they were 2-5 cm long. Rooting occured in 7-10 days (Figure 1c). The rooting was 100 percent and the average ratio of root length to shoot length was 2:1. Eighty rooted plants obtained from the different treatments (Table 3) were successfully hardened in the growth chamber (Figure 1d).

About forty percent of the plants flowered *in vitro* in the rooting phase itself and a few others flowered very early in the field in about 7 days. The rest flowered after 15 days of transfer. The size of flower heads in all the plants was small (about 5cm dia) as compared with the field grown plants of this cultivar (20 cm dia). Seed set was found to be 100% in all these plants. The number of seeds obtained from such plants, the R1 generation, ranged from 2-10 seeds per head. These seeds showed 100% germination.

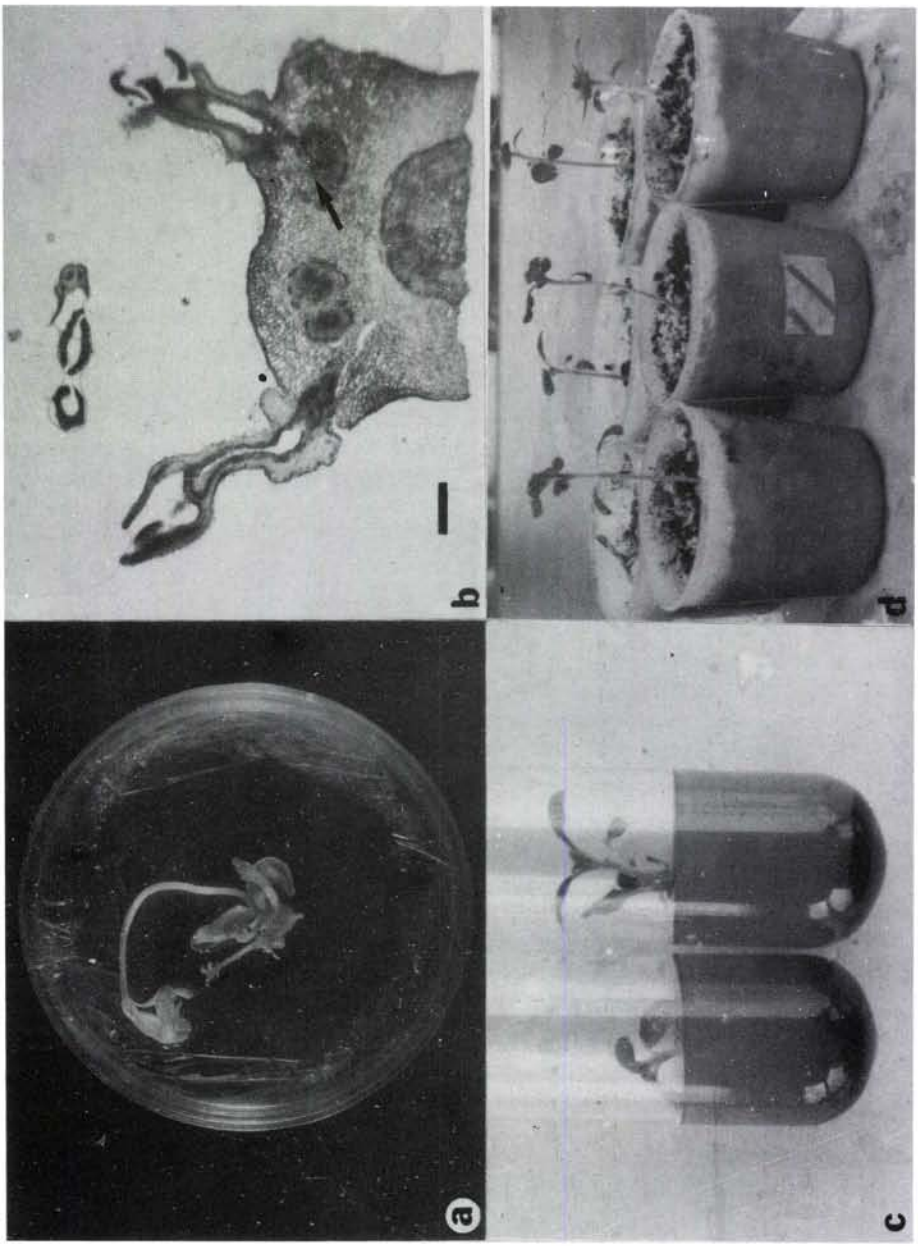

Figure 1: Plant regeneration through multiple shoots from immature embryos of sunflower.
a) Germinated 7 day old embryo with three adventitious shoots.
b) Histology of the adventitious shoots from the hypocotyl region. Arrow: vasculature of adventitious shoot. (Bar = 500 um)
c) Excised shoots cultured for rooting in charcoal-containing medium.
d) Hardened plantlets.

DISCUSSION

Our results indicate that cytokinin-containing medium induces multiple adventitious buds in immature sunflower embryos. In an earlier experiment, only callus was induced when the embryos were inoculated in medium containing even low concentrations of 2,4-D, supporting the fact that sunflower has a high endogenous auxin level (Nataraja and Ganapathi, 1989; Jeannin & Hahne, 1991; Power, 1987; Pugliesi et al., 1991; Rogers et al., 1974; Liu and Reid, 1992). The adventitious bud induction medium used by Jeannin and Hahne (1991) and Freyssinet and Freyssinet (1988) also contained no auxin. While the former reported 70% multiple bud induction response of which only one bud could be grown into a plant, the latter reported 65% response with about one-fourth of the buds becoming hardened plants. In this study the best treatment gave 70% multiple shooting response and more than 65% of the induced buds could be transferred successfully to the field. An additional subculturing step after 10-15 days of bud induction may have helped to improve the number of buds that could be matured. The problem of apical dominance was thus overcome although the buds had to be matured to a height of 2-5 cm still attached as clumps to the hypocotyl tissue, after which they could be separated and grown into individual plantlets. Freyssinet and Freyssinet (1988) obtained 110 buds from 160 explants for T76B cultivar of which only 16 plants proved fertile. In contrast, in our study we obtained two buds/embryo (using 7-9 day old embryo) and all the 80 plantlets produced from the various sizes of immature embryos were fertile.

The structures forming directly on the hypocotyl of zygotic embryos are adventitious vegetative buds and not somatic embryos as confirmed by histological observations. From Figure 1b, it is quite evident that the vasculature of the adventitious buds and that of the hypocotyl is common and the epidermis is continuous unlike the evidence shown by Pelissier et al. (1990) for somatic embryos formed on thin epidermal layers of an inbred sunflower line (HA 300B). The directly forming green shoots/embryoids from the hypocotyl of cultured immature zygotic embryo were, however, considered by Jeannin and Hahne (1991) as direct somatic embryogenesis although histological evidence was lacking.

Induction of adventitious buds was high in 5 and 7 day old embryos (Table 2), after two weeks of initiation, whereas the actual number of plants obtained was higher in 7 and 9 day old embryos (Table 3). Thus the age of the embryo cultured was critical for plant regeneration. Yueng et al. (1981) and Pugliesi et al. (1990) have suggested that explants with many meristematic and undifferentiated cells as in immature stomatal complexes are generally predisposed to form shoots probably because this type of meristematic tissue make the cells responsive to hormonal treatment. From this study it is clear that immature embryos of sunflower (3-11 days after pollination) have sufficient cells that are predisposed to form multiple shoots; the younger embryos, however, show a quicker response suggesting their higher responsiveness to exogenous hormones. The older embryos, though slow to show initial response, generally produce more multiple shoots.

In the field-grown plantlets, maturity period of the plant depended on the leaf number, which ensured better flowering and plant height (Carter, 1978). In our study, the vegetative phase of the embryo-derived plants was very short resulting in early

flowering and small sized flower heads. The influence of the growth hormones and sugar during *in vitro* culture continues in the field-hardened plants so that plants with a height of only 12-25 cm produce flower heads. Early flowering response in cultured sunflowers has also been reported by Henrickson (1954) and Freyssinet and Freyssinet (1988) and is thought to be induced by a decrease in sucrose concentration during the maturation phase of individual plantlets.

The total period taken from culture initiation to obtaining mature seed was 105 days in this experiment. The normal seed-to-seed generation time for the sunflower hybrid variety is 110 days. If seed dormancy period of about 50 days and saving in the seed maturation time of about twenty-five days is also taken into account, there is a total saving of as much as 60-70 days per breeding cycle. The embryo derived plantlets being fully fertile can also lend themselves to further crossing and immature embryo culture. Thus the total crop improvement period can be substantially reduced if this technique is employed in every breeding generation.

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ACORTAMIENTO DEL CICLO DE MEJORA MEDIANTE CULTIVO DE EMBRIONES INMADUROS EN GIRASOL *Helianthus annuus*

RESUMEN

Los embriones inmaduros de hibridos de girasol fueron cultivados en un medio MS suplementado con BAP en el rango de 0.5 a 2.5 mg/l. Las yemas adventicias del tallo fueron inducidas en la región del hypocotilo de los embriones inmaduros de un cruce cutre una linea comercial androesteril (234A) y una linea restauradora (R F1). Estas yemas adventicias. Fueron desarrolladas a tallos enraizados y transferidas al campo fueron obtenidas plantas fertiles con semillas en aproximadamente dos meses y medio desde la iniciación. Este medio ayudará a acortar en tiempo de una generación y por tanto puede ser utilizada provechosamente en programas de mejora del girasol.

RACCOUREIR LE CYCLE DE SÉLECTION DU TOURNESOL (*Helianthus annuus* L.) GRÂCE À LA CULTURE D'EMBRYONS IMMATURES

RÉSUMÉ

Des embryons zygotiques, immatures d'hybrides de tournesol ont été cultivés sur le milieu MS complémenté par du BAP dont la concetration variaient de 0.5 à 2.5 mg/l. Des bourgeons de pousses adventices ont été induits dans la région de l'hypocotyle d'embryons immatures issus de croisements entre une lignée commerciale mâle sterilé (234A) et un restaureur (RF1). Ces bourgeons adventices ont donné naissance à des pousses, ont raciné et ont été transférés en champ. Des plantes fertiles ont été obtenues et donné des graines deux mois et demi aprés l'initiation. Cette méthode aidera à diminuer la durée d'une génération et de ce fait peut être profitablement utilisé dans des programmes de sélection de tournesol.