RAPID REGENERATION OF DIHAPLOID WINTER RAPESEED

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Plant breeders are employing dihaploid breeding techniques with increasing frequency to produce homozygous lines of rapeseed (Brassica napus L.). Standard dihaploid technique involves culturing microspores in a liquid growth media to induce them to develop into a haploid embryo. Once embryos have developed to the cotyledonary stage, they are plated on solid media to develop into haploid plantlets. Diploids are produced by unreduced gametes, endomitosis, chromosome doubling during secondary embryogenesis, or by the application of the chemical colchicine (Beversdorf et al. 1987).

Somaclonal variation, resulting from chromosome abnormalities, can occur as the embryos are subcultured to produce normal plantlets. The frequency of somaclonal variation increases as the time the plant material is maintained in culture increases and is also genotype specific. Somaclonal variation may reduce the vigor of dihaploid lines by introducing deleterious mutations (Baenziger et al. 1984). Dihaploid lines produced from 'Tower' revealed morphological variants, reduced vigor, sterility, and segregating progeny for glucosinolate levels (Orton 1984).

The objective of our research was to develop a method of regenerating embryos into plantlets faster, less expensively, and with less somaclonal variation. Our rapid regeneration procedure eliminates the need to use solid media by planting the embryo directly into potting soil. This eliminates approximately six months in the production of dihaploid seed from the initial cross of the parents. The rapid regeneration system was based on the hypothesis of treating the embryo as an emerged seedling since the seedling and the embryo are anatomically very similar. Successful establishment of the embryo under these conditions is dependent upon the maturity of the embryo and the environment in which it is placed. This paper reports experiments conducted to optimize embryo age and environment.

MATERIALS AND METHODS

Donor plants of Brassica napus L. cv. 'Rebel' were grown to flowering in a growth chamber set at 16 h photoperiod, 15/10°C day/night temperature regime (Kellar et al. 1987). Buds 2.5 to 4 mm in length were harvested, surface sterilized and macerated to isolate microspores by passing the slurry through 44 micron filters. The microspores were washed four times in Gamborg's B_5 liquid medium (13% sucrose), resuspended in modified NLN liquid medium with 13% sucrose, and then incubated at 32°C in the dark for three days. Embryos were then transferred to 25°C until they were 21 to 35 days in age (Polsoni et al. 1984). As soon as embryos were visible, they were placed on a rotary shaker set at 50 rpm. This improved the number of torpedo shaped embryos (Coventry et al. 1988; Beversdorf et al. 1987). Embryos were fully mature when 4 to 8 mm in length, approximately 21 to 28 days after culturing. Since the time required to reach this stage was directly dependent upon nutrient competition, replenishing the media with a lower concentration of sucrose (8% instead of 13%) at three weeks was beneficial.

Mature embryos were planted in clear plastic bakery containers, approximately 20 x 25 cm with hinged lids. These containers were used as mini-greenhouses to maintain high humidity, micro-environments for the

embryos once they were planted. Ventilation and drainage holes were punched in the top and bottom, before they were filled with Sunshine^{R} brand potting mix. Once the embryos were planted, the containers were placed in a growth chamber set at 19/16°C (day/night), 12 h photoperiod, a relatively low light intensity (120 μmoles m^2 sec^{-1}), and 80-90% RH. Rinsing the sucrose medium off the embryos before planting lessened the fungal contamination. If fungal contamination did occur, a light (5%) Benylate mist usually controlled the problem.

Pre-plant Embryo Treatment

Four pre-plant treatments were given to three week old embryos and the results were compared to embryo regeneration using standard procedures on solid B_5 medium with 2% sucrose without plant hormones. All other treatments involve planting the embryos into soil after a pre-plant treatment. These treatments included planting embryos grown in liquid culture and kept in the dark, planting embryos grown in liquid culture and exposed to low light intensity, planting embryos into soil after five days on solid B_5 media to enhance root development, and planting embryos into soil after five days on solid B_5 media with exposure to low light intensity to enhance both root and chlorophyll development. All containers were planted and placed in the growth chamber the same day. Embryos were randomly selected for each treatment from a uniform culture taken from the same donor plants.

Embryo Age

In a separate experiment, the effect of age on embryo establishment was determined. Embryos from a uniform culture taken from the same donor plants were planted at three, four and five weeks of age. A random selection of embryos at each age eliminated embryo size as a variable. Those embryos left in liquid media were replenished with fresh media to ensure vigorous, continuous growth. The containers of planted embryos were placed in a greenhouse with 16 h photoperiod under approximately 21/13°C day/night temperature regime. Midwinter weather conditions at Moscow, ID, are mostly overcast, with very little direct sunlight.

RESULTS AND DISCUSSION

Pre-plant Embryo Treatments

Approximately 85% of the embryos treated with liquid/dark treatment prior to planting survived and developed into plantlets with true leaves (Fig. 1). True leaves emerged from the primary meristem in the center of the fused cotyledons within two to three weeks after planting. If true leaves did not develop by four weeks the embryo usually died. Very few embryos treated with the liquid/light treatment or the solid/dark treatment survived. Embryos placed on solid media under lights for one week prior to planting were more successful in establishment because of secondary embryogenesis, which developed along the hypocotyl when embryos were placed on solid media. These secondary embryos competed with the primary meristem and delayed leaf formation. The secondary embryogenesis also caused double stems. No secondary embryogenesis was observed in the liquid based pre-treatments. Neither root development on solid media nor exposing the embryos to light prior to planting enhanced survival.

Embryo Age

Embryo maturity was found to be a critical factor. Survival of four and five week old embryos was greater than those at three weeks. The older the embryo at planting, the faster the true leaves developed and the quicker the embryo became established (Table 1). Embryos treated with standard regeneration procedures were still being subcultured and no

survival data was available five months after the initiation of the experiment.

Up to 50 to 60% of the embryos placed in the greenhouse were able to establish, although the conditions in the greenhouse were much more variable. Condensation within the plastic containers under these conditions increased the humidity and fungal contamination caused significant losses. Ventilation in the growth chambers eliminated the condensation problem in the earlier experiment.

Although the mini-greenhouses created a high-humidity environment that promoted fungal growth, this high humidity was necessary for the survival of the embryos. Similar results were obtained when cauliflower plants (Brassica oleracea var. botrytis) developed reduced levels of epicuticular wax in tissue culture (Grout et al. 1977). These plants experience excessively high levels of water loss when transplanted from a liquid environment to a drier terrestrial environment. High humidity levels were required to reduce water loss while the embryos adapted to the soil.

CONCLUSIONS

Based upon these results, we feel that it is possible to speed up the regeneration procedure for recovering haploid plantlets by planting 4 to 5 week old microspores directly into soil. The results of these experiments compare favorably with the number of plantlets eventually recovered from standard subculture and transplanting procedure. The rapid regeneration procedure produces plantlets 4 to 6 months faster than standard subculturing and requires less labor and supplies. These containers also provide an efficient system for vernalization since each 20 x 25 cm container easily holds 80 to 100 embryo derived plantlets. Additional experiments are evaluating the genotype specificity of this procedure and determining if a correlated selection response exists for increased vegetative vigor when plantlets are transplanted directly into soil.

REFERENCES

BAENZIGER, P.S., KUDIRKA, D.T., SHAEFFER, G.W. and LAZAR, M.D. 1984. Significance of doubled haploid variation. In: Gene Manipulation. J.P. Gustafson (ed.). Plenum Press, New York. pp. 385-414.

BEVERSDORF, W.D., CHARNE, D.G., KOTT, L.S., CHUOUNG, P.V., POLSINIM, L. and ZILKA, J. 1987. The utilization of microspore culture. Proceedings of Seventh International Rapeseed Congress, Poznan, Poland. pp. 86-91.

COVENTRY, J., KOTT, L.S. and BEVERSDORF, W.D. 1988. Manual for microspore culture technique for Brassica napus. Dept. of Crop Science Technical Bulletin, OAC Publication 0489. pp. 28-29.

GROUT, W.W. and ASTON, M.J. 1977. Transplanting of cauliflower plants regenerated from meristem cultures. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hort. Res. 17: 1-7.

KELLAR, W.A., ARNISON, P.G., and CANDY, B.J. 1987. Haploids from gametophytic cells-recent developments and future prospects. In: Plant Tissue and Cell Culture. Alan R. Liss, Inc. pp. 223-241.

ORTON, T.J. 1984. Somaclonal Variation: Theoretical and Practical Considerations. In: Gene Manipulation. J.P. Gustafson (ed.). Plenum Press, New York. pp. 421-468.

POLSONI, L., KOTT, L.S. and BEVERSDORF, W.D. 1988. Large scale microspore culture technique for mutation and selection studies in Brassica napus. Can. J. Bot. 66: 1681-1685.

SCOWCROFT, W.R. and LANKIN, R.J. 1981. Somaclonal variation-a novel source of variability from cell cultures for plant improvement. TAG 60: 197-214.

SIEBEL, J. and PAULS, K.P. 1989. A comparison of anther and microspore culture as a breeding tool for Brassica napus. TAG 78: 474-479.

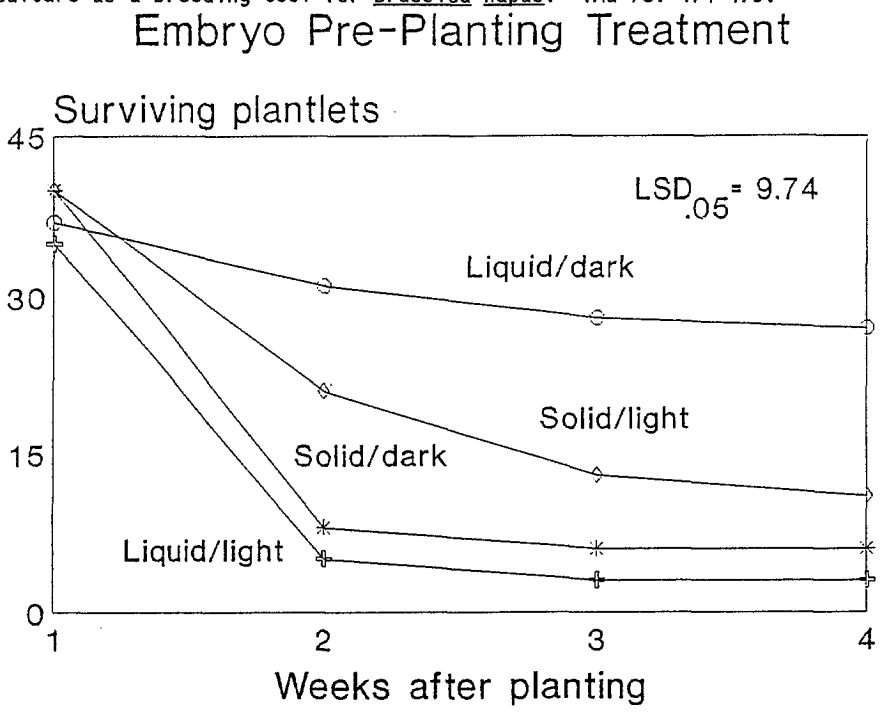

Figure 1. Number of surviving plantlets following transplanting 3 week old embryos into soil with four pre-treatments.

Table 1. Percent of survivors and days to formation of first true leaves of embryos of B. napus 3, 4, and 5 weeks after culturing.

Embryo Age	Embryo Survival	First True Leaf
	--%--	---Days---
3 weeks	20	25
4 weeks	53	21
5 weeks	68	17
$LSD_{(0.05)}$	7.05	--