DEGREENING AND ITS INHIBITION BY STRESS IN HAPLOID EMBRYOS OF BRASSICA NAPUS CV TOPAS AND JET NEUF

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INTRODUCTION

Green seed causes a major production problem in canola, because the oil produced from such seed is green. This green colour must be removed from the oil through bleaching, a process which significantly reduces the oil yield. Therefore, the value of a crop is reduced when there is as little as 6% green seed.

In the northern reaches of the canola growing regions, plants are frequently exposed to frost during seed development and this inhibits seed degreening. Thus our goal was to select for superior seed degreening following frost. As haploid embryos are known to mimic many of the developmental changes in the zygotic embryo, we felt that they may be a useful system for the selection and study of degreening. The present study describes a greening and degreening protocol for haploid embryos. With this system, we compared the effect of stress between haploid and zygotic embryos.

MATERIALS AND METHODS

Plant material and freezing protocol

B. napus cv. Westar, was used as the control material. Plants were grown, frozen and sampled according to previously published methods (Johnson-Flanagan et al., in press).

Haploid embryo production

The methods for microspore isolation and haploid embryo development have been described elsewhere (Orr et al., 1986). The embryos were grown in the dark at 25^{0}C for 18 days, then shaken at 80 rpm for an additional week in the dark, at room temperature.

Greening and degreening protocols

Approximately 29 days after isolation, the embryos were greened under lights (250 $\mu E^{-2}m^{-1}$) for 7 days. They were then subcultured into fresh medium containing ABA ranging from 0 to 20 ppm and sucrose ranging from 6.5% to 20%, and degreened in either the light or dark.

HPLC

Methods for pigment extraction and analysis have been described in detail elsewhere (Johnson-Flanagan and Thiagarajah, 1990).

Nucleated freezing and supercooling

Embryos were washed 3 times with distilled water, placed on moist filter paper and either nucleated at -2^{0}C as previously described (Orr et al., 1990), or left to supercool. The minimum temperature reached was -5^{0}C, and the rate of cooling was 5^{0}C/h (Orr et al., 1990). Embryos frozen in sucrose were washed as above, then placed in petri dishes containing 15 ml 13% sucrose and nucleated as above. In all cases, embryos were allowed to thaw at 4^{0}C overnight in dark.

Regeneration was assessed as above.

NMR

Fifteen seeds were placed in a 5 mm NMR tube. Shimming was done on 30% ethylene glycol in D_2O. The spectra were acquired with a Bruker AM 250 at a frequency of 250 MHz.

Desiccation

Embryos were desiccated by passing them through a series of moisture contents over a 72 hour period. The moisture contents were maintained in sealed desiccators, using the saturated solutions outlined by Senaratna et al. (1989).

RESULTS

Greening and degreening protocol

The degreening protocols are outlined in Figure 1. In all cases, degreening in the dark was more effective than degreening in the light. The optimum conditions for dark degreening were 13% sucrose and either no ABA or 5 ppm ABA. Degreening in the light was stimulated by the addition of ABA, with 5 ppm being more effective than 10 ppm.

Pigment analysis

Green haploid embryos contained primarily chlorophyll a and b with a small amount of pheophytin a and trace amounts of chlorophyllides, pheophorbides and pheophytin b (Table 1). Degreening led to pigment losses over the 15 day period (Fig. 2a). In comparison, the zygotic embryos contained primarily chlorophyll a and b, and to a lesser extent, pheophytin a (Table 1). The pattern of degreening is shown in Figure 2b.

Exposure to subzero temperatures

Nucleated freezing at -2^0 or -5^0C resulted in haploid embryo death. Attempts to increase the tolerance through exposure to 4^0C for 3 days failed to increase the survival. On the other hand, exposure of haploid embryos to nonnucleated freezing up to -5^0C had no effect on survival. Furthermore, the degreening potential of the embryos was not diminished.

Nucleation temperature in the zygotic embryo

The temperature at which ice formed in the zygotic embryo was determined by NMR (Fig. 3). Ice nucleation in seed in the 70% moisture range occurred below -5^0C and was complete at -10^0C. The nucleation temperature shifted to between -15 and -20^0C in seed in the 50% moisture range.

Seed moisture content

Sublethal freezing caused a very significant reduction in the seed moisture content within days of the stress (Fig. 4). This was not an immediate response to freezing as the moisture content remained the same as the control for the first 24 hours following the freeze.

Desiccation

Exposure of the haploid embryos to rapid desiccation at various stages of degreening fixed the amount of pigment present in the embryo. The pigments in the embryo were analysed by HPLC and compared to results obtained from mature seed that had been subjected to -5^0C during development (Table 2).

DISCUSSION

Haploid embryos have been viewed as a powerful selection system for crop improvement in canola. However, not all

characteristics are amenable to the application of haploids. For example, under standard culture conditions, haploid embryos do not accumulate storage proteins (Taylor et. al., 1990). On the other hand, we recently demonstrated that haploid embryos can attain freezing tolerance and the degree of tolerance reflects the tolerance in planta (Orr et al., 1990). The haploid embryos may be very useful in selection for superior oil quality (Talor et. al., 1990) as both the developmental sequence of deposition and the lipid characteristics are very similar in the zygotic and haploid embryos. In the present study we have extended the utility of haploid embryos to include selection and study of degreening and freezing tolerant degreening in the seed.

Results from the present study show that the best degreening protocol involved exposure to high osmoticum and ABA. Similar conditions have been shown to be essential for normal embryo development in vitro (Crouch and Sussex, 1981, Finkelstein and Crouch, 1986; Finkelstein et al., 1985) and for artificial seed development (Senaratna et al., 1989).

Sublethal freezing is known to inhibit seed degreening. However, before an in vitro selection system can be implemented, it is necessary to determine the mechanism by which frost affects the seed. To this end, we examined ice formation within the seed and the effect of supercooling on haploid embryos. The results indicate that ice formation may not be a factor, as ice did not form in isolated seed. Further, supercooling failed to impact on degreening. Finally, we examined the effect of desiccation on degreening in haploid embryos, as our research indicated that seed undergoes rapid desiccation following freezing. We concluded that rapid desiccation can be used in vitro to mimic the effect of exposure to freezing temperatures.

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Table 1. Percentage of pigments in green haploid embryos and zygotic embryos. Values are the means of at least 5 determinations ± SE.

Pigments	Haploid embryo	Zygotic embryo
	%	(75-80% seed moisture)
chlorophyllide a	.65+ .4	.6 + .2
chlorophyllide b	1.1 + .8	1.0 + .4
pheophorbide a	trace	trace
pheophorbide b	trace	trace
chlorophyll a	57 + 4	69 + 5
chlorophyll b	36 + 2	28 + 3
pheophytin a	4 +2.5	.6+ .2
pheophytin b	trace	trace

Table 2. Pigments in haploid embryos following rapid desiccation during degreening as compared to zygotic embryos frozen during degreening. The haploid embryos were rapidly desiccated following 5, 10 or 15 days of degreening. The zygotic embryos were frozen at the moisture contents noted, and allowed to mature prior to analysis (data from Johnson-Flanagan et al, 1990). Values are the means of at least 4 determinations.

Pigments	5	10	15	65%	56%	52%
				%		
chlorophyllide a	2	3	7	1	3	0
chlorophyllide b	0	0	0	0	0	0
pheophorbide a	0	0	0	63	23	14
pheophorbide b	0	0	0	0	1	0
chlorophyll a	78	78	83	9	42	60
chlorophyll b	16	16	10	10	18	26
pheophytin a	4	3	0	14	12	0
pheophytin b	0	0	0	0	0	0
Total (ng/embryo)	682	575	41	743	722	23

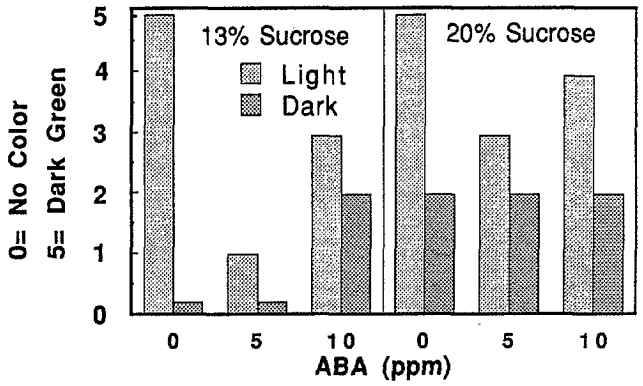

Fig. 1.Degreening of haploid embryos.

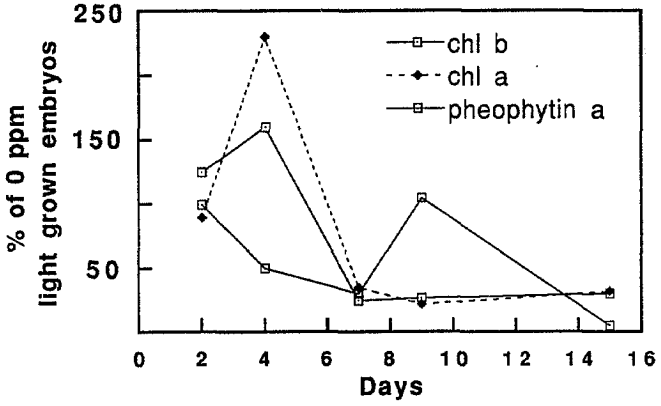

Fig.2a.Pigment changes during degreening of haploid embryos.

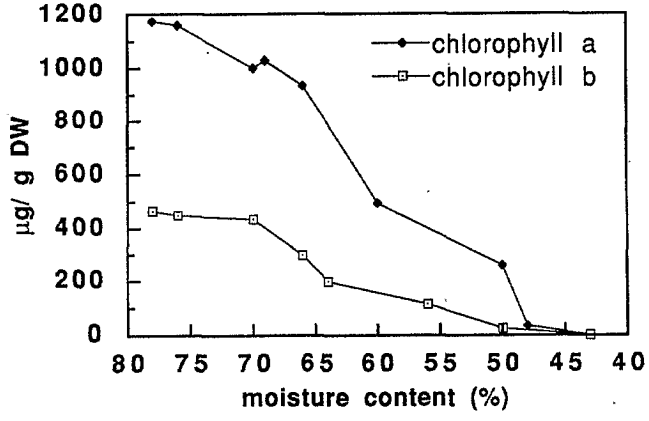

Fig.2b.Pigment changes during degreening of zygotic embryos.

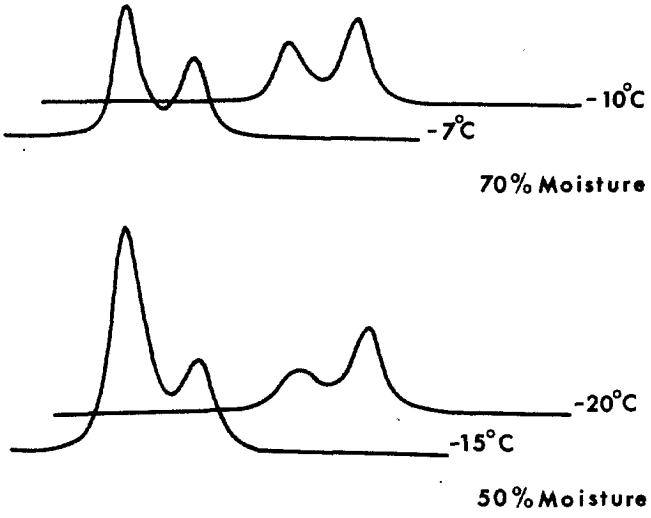

Fig. 3.NMR scans showing ice formation in seeds.

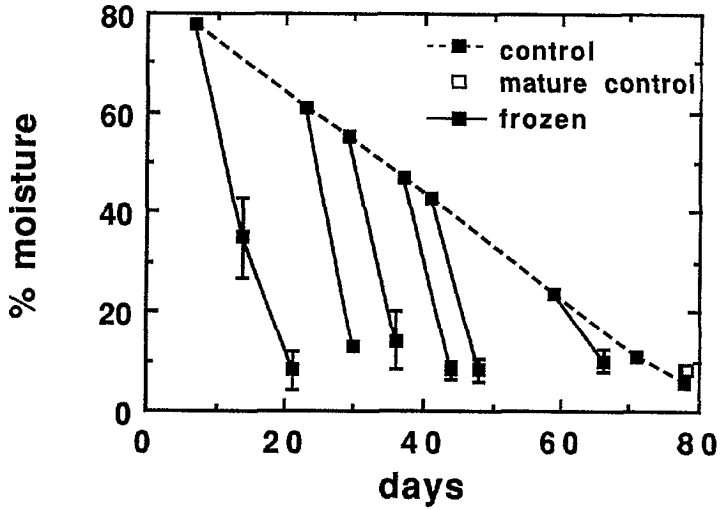

Fig. 4.Change in seed moisture content following freezing.