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THE INFLUENCE OF LOW TEMPERATURE TREATMENT ON GROWTH AND PROLINE ACCUMULATION IN POLYEMBRYOGENIC CULTURES OF OIL PALM (*Elaeis guineensis* Jacq.)

Keywords: Low temperature; oil palm polyembryogenic cultures and proline accumulation

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Low temperatures (15°C–20°C) were observed to inhibit the growth and multiplication of oil palm polyembryogenic (PE) cultures. These conditions enable PE cultures to be kept for at least six months without subculturing. The method can, therefore, be exploited for minimal growth storage of in vitro *cultures. Proline, which is known to be a universal stress indicator, was found to accumulate in the cultures under low temperature stress and the increase was about two-fold during 30 days of exposure. In the presence of 0.5 M sucrose, low temperature storage of cultures could be extended even to nine months.*

INTRODUCTION

The maintenance of tissue cultures under growth-limiting conditions offers possibility of reducing the requirement for subculturing (Withers, 1984). Some of the techniques employed to achieve this include the use of low temperature, minimal growth media and modification of the gaseous environment (Stushnoff and Fear, 1985). Each of these methods offers a valuable facility for a particular laboratory situation (Withers, 1984), but the most common and promising method for limiting growth is maintaining the cultures at a reduced temperature.

Some of the growth-limiting treatments, besides suppressing growth and multiplication, may also induce stress conditions in the cultures. For example, low temperature incubation may induce chilling stress, high osmotic conditions could induce moisture stress (dehydration), and total immersion of cultures in non-agitated liquid media may induce flooding effects or anaerobic

stress. As most of the growth-limiting treatments, including low temperature storage, induce stress, identification of a biochemical or physiological indicator would be beneficial in monitoring the behaviour of treated cultures.

Proline in plant cells and tissues is a known stress indicator and it has been reported to accumulate under various stress conditions (Aspinall and Paleg, 1981). In this study we examined the effect of low temperature storage on growth and accumulation of proline in polyembryogenic (PE) cultures of the oil palm.

MATERIALS AND METHODS

The polyembrogenic (PE) cultures used for this study were taken from oil palm clone P10 (PORIM's clone 10), a highly prolific producer of embryoids. The cultures were propagated continuously on modified Murashige and Skoog (MS) media (Paranjothy *et al.*, 1989) and maintained at 27°C–29°C, with a 12 hr photoperiod, 1000 lux light intensity (fluorescent illumination: 90 µmol m-2s-1 PAR) and relative humidity at about 60–70 per cent. The cultures were subcultured at 3–4 month intervals.

Low temperature treatment and analysis of growth

As a preliminary observations had shown that 15°C–20°C was suitable for storage of oil palm cultures (Paranjothy *et al.*, 1989), a similar temperature regime was used throughout these studies. Embryoids were cultured on to MS media + 3% sucrose + 5 µM IBA (3-indolebutyric acid) and stored at 15°C, 20°C or 27°C (ambient, control temperature).

The following measurements were made at two-month intervals over a six-month study period:

a. Fresh weight.
b. Dry weight – This was determined by drying the materials at 105°C for at least 16 hr or until a constant weight was obtained.
c. Number of shoots.

Some of the embryoids (at least 12 clumps) from low temperature treatments were transferred every two months to the normal conditions (27°C) for observations on their survival and capacity for regeneration. After the three samplings at intervals of two months the treated cultures were kept for a further of six months and observations on their physical responses to the low temperature were made.

Proline estimation

Embryoids were cultured on MS media (as described above), incubated at 15°C and 20°C and analysed for proline content at intervals of 10, 20 and 30 days. Similar analyses were also conducted at hourly and two day intervals on cultures incubated at 15°C.

Proline was determined by the method of Bates *et al.* (1973). Approximately 0.5 g of plant material was homogenized in 10 ml of 3% aqueous sulphosalicylic acid and the homogenate filtered through Whatman No. 2 filter paper. Two ml of the filtrate was mixed with 2 ml acid-ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid), 20 ml 6M phosphoric acid, and 2 ml of glacial acetic acid in a test tube, which was kept for one hour at 100°C; the reaction was terminated by cooling in an ice bath. The reaction mixture was extracted with 4 ml toluene by mixing vigorously with a test tube stirrer for 15–20 seconds. The chromophore-containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene as a blank. The proline concentration was determined from a calibration curve using L-proline as a standard, and calculated on a fresh weight basis.

Treatments with exogenous sucrose and proline

The embryoids were cultured on MS media with added sucrose to a final concentration of 0.5M, or added proline (10mM). The cultures were incubated at 15°C and 20°C. The concentrations of sucrose and proline used were based on the effective concentrations reported by Tarmizi *et al.* (1992). The physical and morphological appearance of the cultures was recorded after seven months of culture.

Statistical analysis

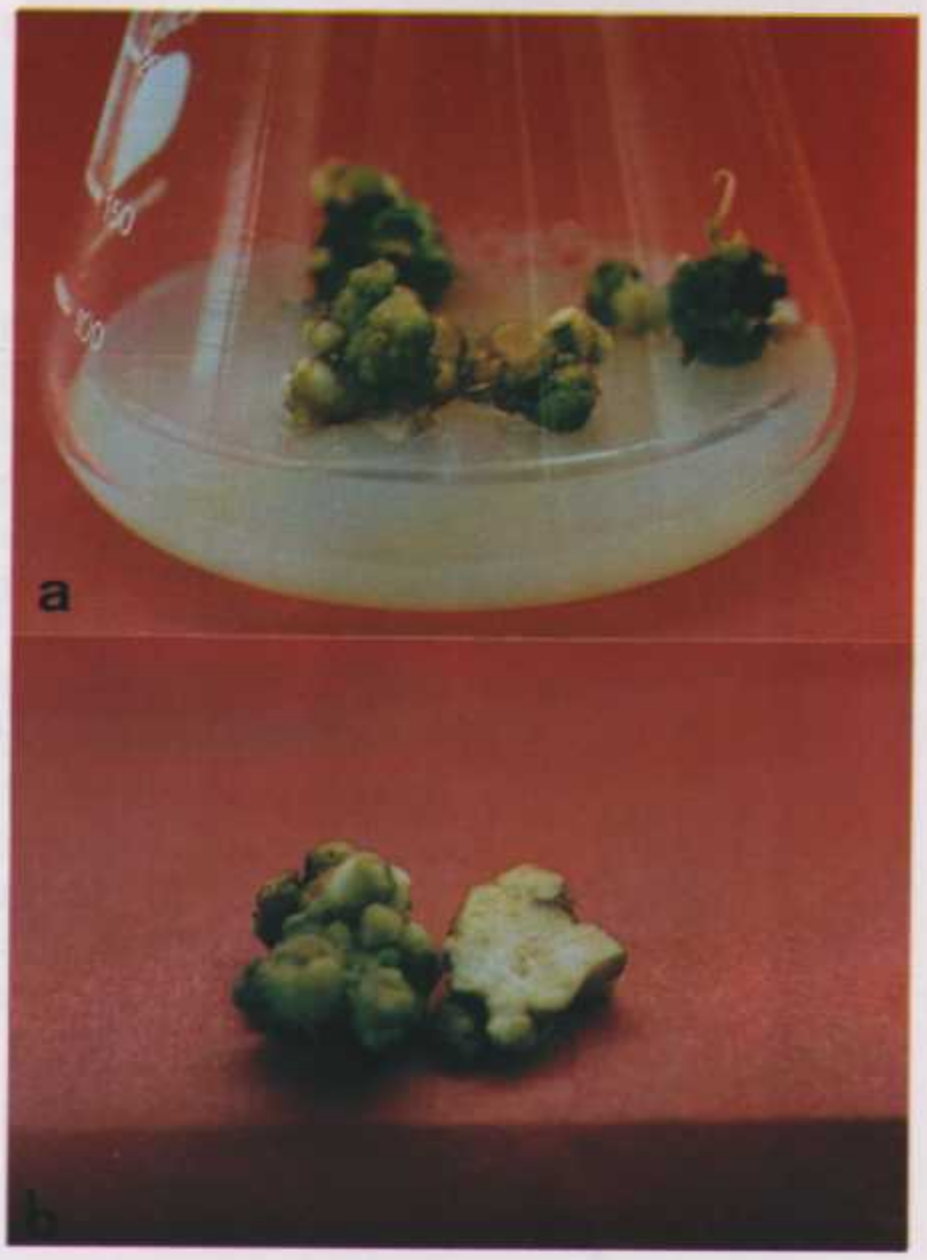
All data collected for this study were means of at least three replicates. Standard error values are presented.

RESULTS AND DISCUSSION

The effect of low temperature on growth and multiplication of oil palm polyembryogenic (PE) cultures

Studies on the effect of six months' low temperature storage on oil palm PE cultures revealed that a temperature of 15°C–20°C inhibited the growth and multiplication of the embryoids (*Figures 1* and *2*). The fresh and dry weights of cultures held at ambient temperature (27°C) increased by 5-fold after three months of culture. However, in low temperature treatments, the increases in fresh and dry weight were between 2 and 3-fold and these cultures continued to grow at a minimal rate throughout the period of investigation (six months) (*Figures 3* and *4*). After three months control treatments were no longer monitored, as the PE cultures differentiated into shoots which covered the whole culture vessel by four months. After four months of growth, the number of small shoots formed in the control cultures was three times and two times higher than those obtained in cultures at 15°C and 20°C, respectively (*Table 1*), and large shoots appeared only in controls. The percentage shoot regeneration after storage at 15°C and 20°C was 67%–79% on transferring to ambient conditions (270°C) and culturing for two months (*Table 2* and *Figure 5*).

Minimal growth storage is one of the approaches used in *in vitro* storage of plant cultures (Stushnoff and Fear, 1985). The

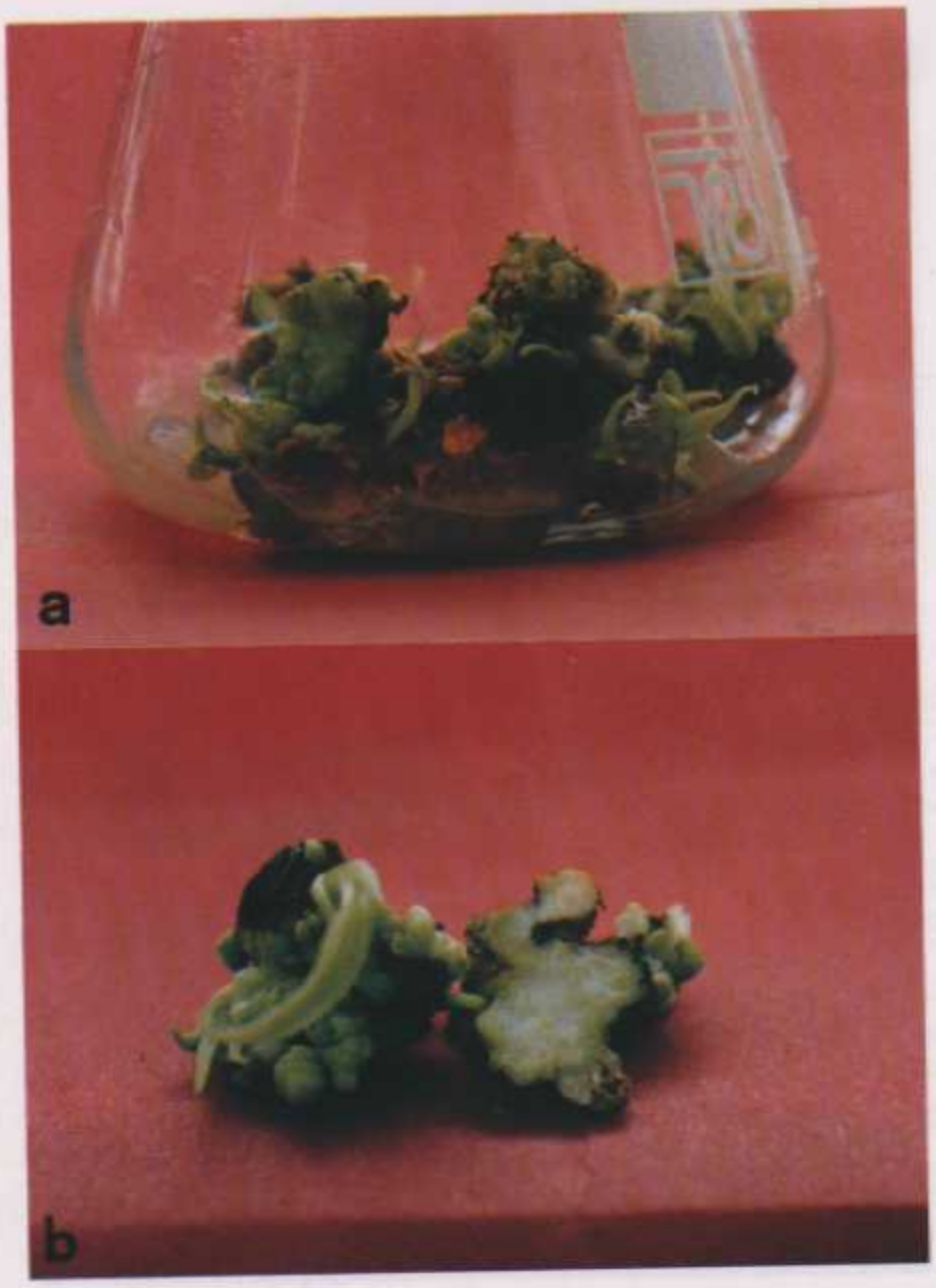


a. In flask (1.3x)
b. Magnified (1.5x)
left: intact, right: cross section

Figure 2. Oil palm polyembryogenic cultures stored at 15°C for six months.

a. Extensive shoot proliferation in culture flask (0.9x)
b. Magnified presentation of some shoots (1.2x)

Figure 1. Six-months old embryoids of oil palm maintained at 27°C (ambient temperature).



a. In flask (1.3x)
b. Magnified (1.7x)
left: intact, right: cross section

Figure 3. Oil palm polyembryogenic cultures stored at 20°C for six months.

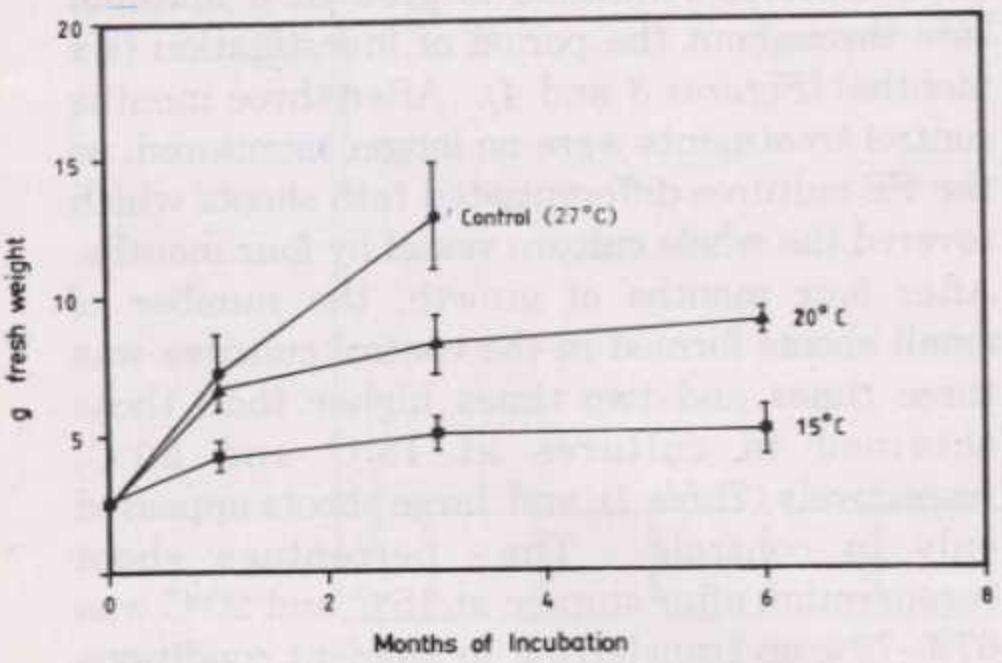


Figure 4. Fresh weight changes in oil palm embryoids cultured at various temperatures. Each point represents the mean of three replicates ± S.E.

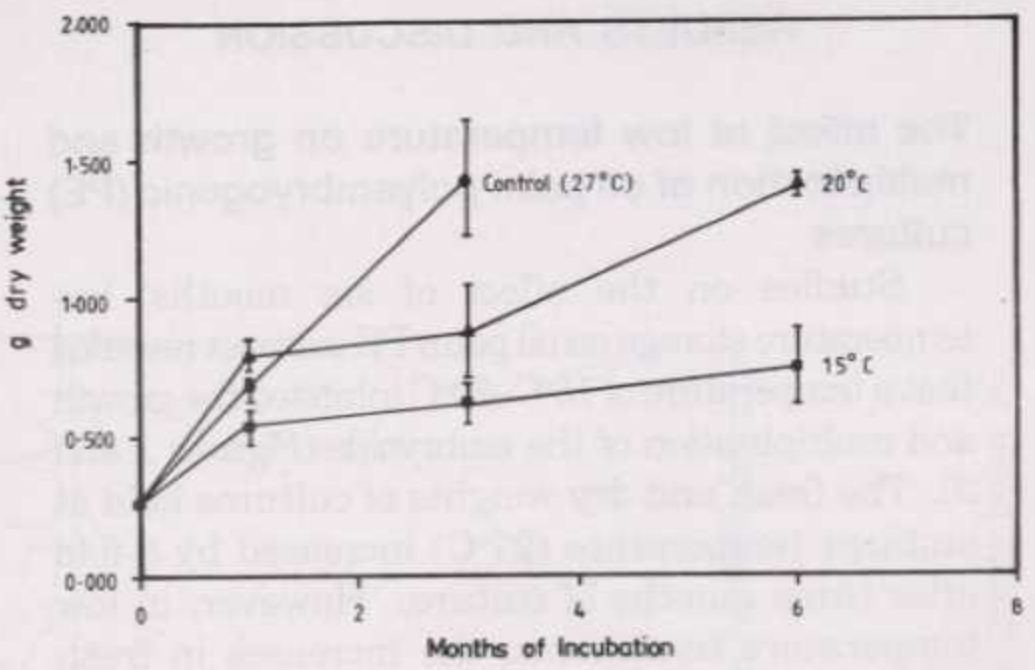


Figure 5. Dry weight changes in oil palm embryoids cultured at various temperatures. Each points represents the mean of three replicates ± S.E.

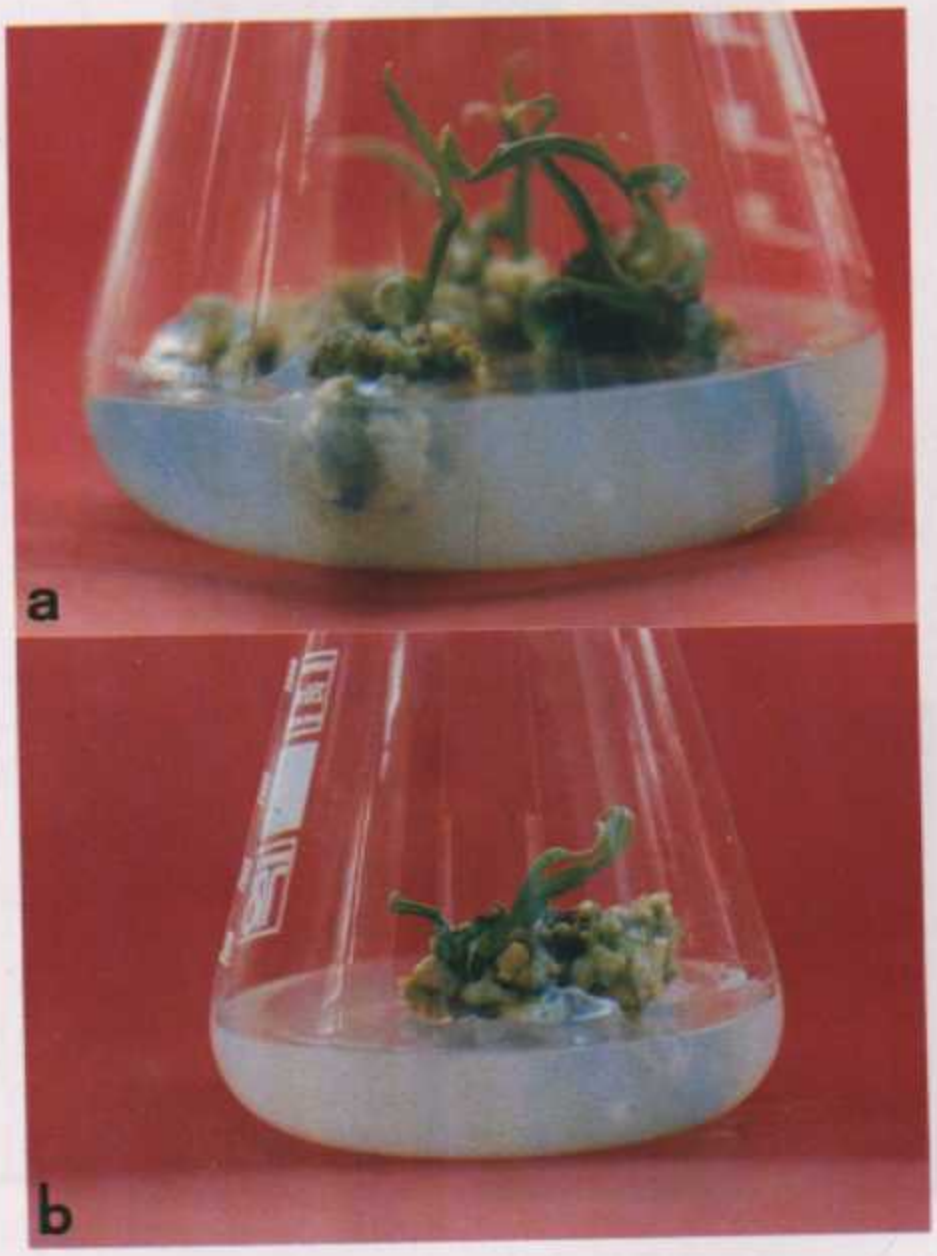


Figure 6. Cultures kept for six months at 15°C (a) or 20°C (b) then regenerated for two months at 27°C (normal conditions) following:

a. 1.3X
b. 0.9X

TABLE 1. SHOOT FORMATION IN CULTURES INCUBATED AT VARIOUS TEMPERATURES

Period in culture (months)	Shoot size	Number of shoots per flask		
		15°C	20°C	27°C (control)
0	Small	0	0	0
	Large	0	0	0
2	Small	0	2.33 ± 0.33	15.3 ± 0.88
	Large	0	0	0
4	Small	4.33 ± 0.32	6.67 ± 0.66	13.33 ± 0.89
	Large	0	0	14.67 ± 1.53
6	Small	5.33 ± 0.34	13.33 ± 0.89	Shoots occupied the whole flask
	Large	0	0	

Small ± < 3 cm; Large ± > 3 cm

objective of these approaches is to extend the subculture intervals, thus enabling the cultures to be maintained for medium term storage. Low temperature storage is one of the minimal growth treatments employed and in this study it was found to be effective in inhibiting growth and multiplication of the oil palm PE cultures. Temperatures ranging between 15°C and 20°C seemed to be suitable for low temperature storage. These conditions considerably reduced increase of fresh and dry weight and shoot regeneration. Cultures could, therefore, be maintained for at least six months without subculturing as compared with the normal subculture cycle for oil palm polyembryogenic cultures, which is three to four months. Embryoids cultured under low temperature storage conditions generally showed prolific growth on transfer to ambient temperatures. Henshaw *et al.* (1982) first suggested that tissue culture material of tropical plant species could be adequately stored at temperatures of 14°C to 18°C, while Withers (1984) proposed higher temperatures between, 20°C to 22°C, as being more suitable, possibly to avoid chilling injury which could affect tropical crops if the temperature was lower than 15°C (Ng and Ng, 1991). The use of low temperature as a method of *in vitro* storage has been used successfully with grapevine (Gazly and Compan, 1988), white clover (Bhojwani, 1981), *Prunus* (Marino *et al.*, 1985) and shoot cultures of several Australian woody species (Williams and Tarji, 1987). Recently, Corbineau *et al.* (1990), in their study on ethylene production in oil palm embryoids, observed that a temperature of

TABLE 2. REGENERATION CAPACITY OF TREATED CULTURES AFTER TRANSFERENCE TO AMBIENT CONDITIONS

Period in culture (months)	Percentage Regenerated Shoots		
	Temperature regimes		
	Ambient (Control)	15°C	20°C
2	100	100	100
4	100	71 ± 4.00	79 ± 5.66
6	100	67 ± 8.50	71 ± 4.00

[1]Monitored 2 months after transfer to ambient conditions. Expressed as % shoots regenerated per 12 clumps.
[2]Overcrowding occurred and plantlets had to be subcultured.

20°C was suitable for suppressing the growth of the cultures and that all the clumps remained viable even after 30 weeks of storage.

In our study it was observed that embryoids stored continuously for more than six months at 15°C or 20°C turned soft and displayed poor growth (*Figure 6*). These cultures were affected by the prolonged exposure to low temperature conditions. Furthermore, biochemical or physiological changes in the tissues may have also indirectly changed the composition of the basal media (Williams and Tarji, 1987) rendering it less suitable for supporting growth. The softening of tissues could also reflect a difference in responses of different genotypes, as reported in grapevine (Barlass and Skene, 1987). Marino *et al.* (1985) observed that the 'age' of the cultures under cold treatment was important in determining the survival and regeneration capacity. Old cultures maintained too long on the same media seemed to show a slowing down in effective growth (Marino *et al.*, 1985) as also observed in this study with oil palm PE cultures.

Figure 7. The softening of embryoid tissues after six months' storage at 15°C (1.5x).

The influence of low temperature stress on proline accumulation in oil palm polyembryogenic cultures

Since low temperature stress inhibited the growth and multiplication of the oil palm PE cultures (*Figures 3* and *4*; *Table 1*) studies were also conducted to examine the possible mechanisms associated with this stress. As already mentioned, one phenomenon that occurs during plant stress is the accumulation of proline (Aspinall and Paleg, 1981). In the present study, it was also found that low temperatures, ranging from 15°C–20°C, induced proline accumulation in the cultures and the increment was 2-fold during 30 days of exposure (*Figure 7*). Proline seemed to start accumulating within 10 hr after exposure to 15°C (*Figure 8*). It continued to increase gradually to about twice the amount found in the control (*Figure 9*) over a period of 8–10 days.

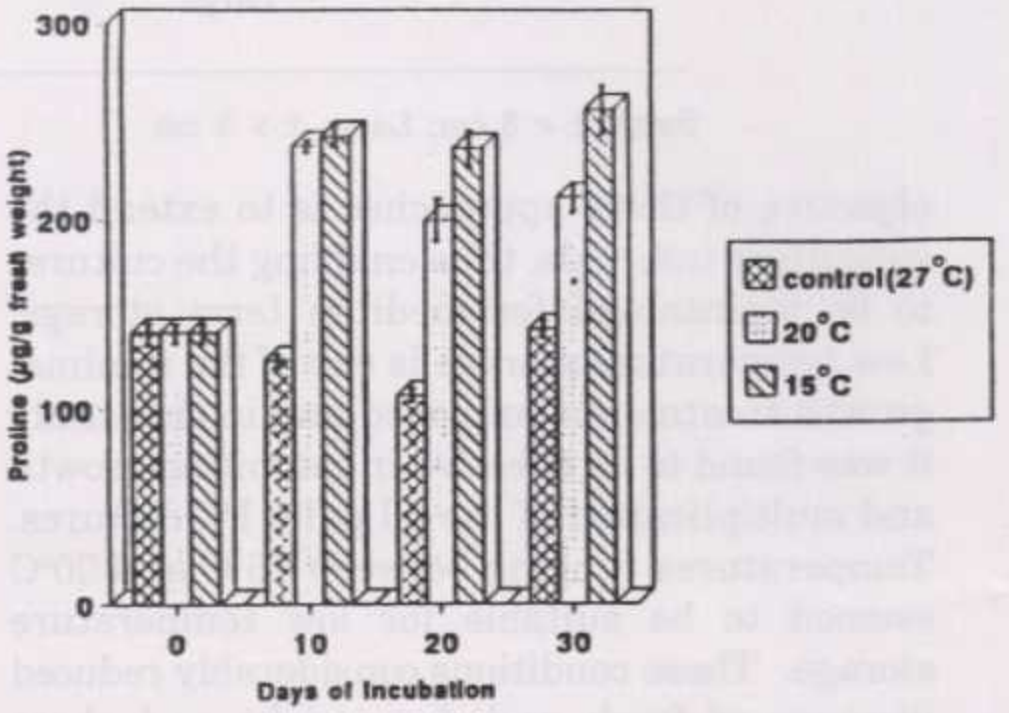


Figure 8. The influence of various temperature treatments on proline accumulation in embryoid tissues. Each histogram represents the mean of three replicates ± S.E.

The proline content in the cultures held at ambient temperatures was around 125 µg/g fresh weight throughout the ten-day period. The accumulation of free proline has also been reported in tissues of other plants after low temperature exposure. Chu *et al.* (1974) found that low temperature caused a reduction in growth, which was accompanied by a marked accumulation of free proline in the leaves of barley. Virgue and Li (1976) reported the accumulation of proline in potato tubers exposed to low temperatures. Free proline accumulation was also observed in the leaves of orange and grapefruit trees grown on a wide range of rootstocks during cold hardening (Yelenosky, 1979; Purvis and Yelenosky, 1982). Proline levels in hardened plants of two winter wheat

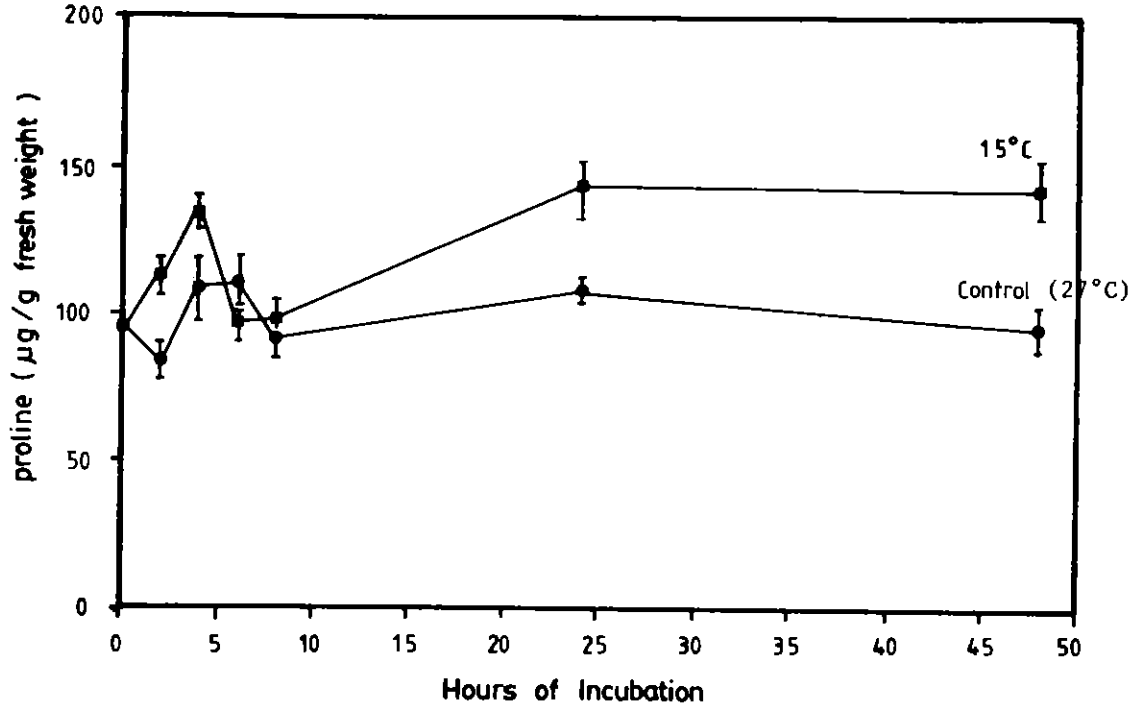


Figure 9. The effect of low temperature (15°C) on proline accumulation over 48 hours of culture in embryoid tissues. The data indicate a mean of three replicates ± S.E.

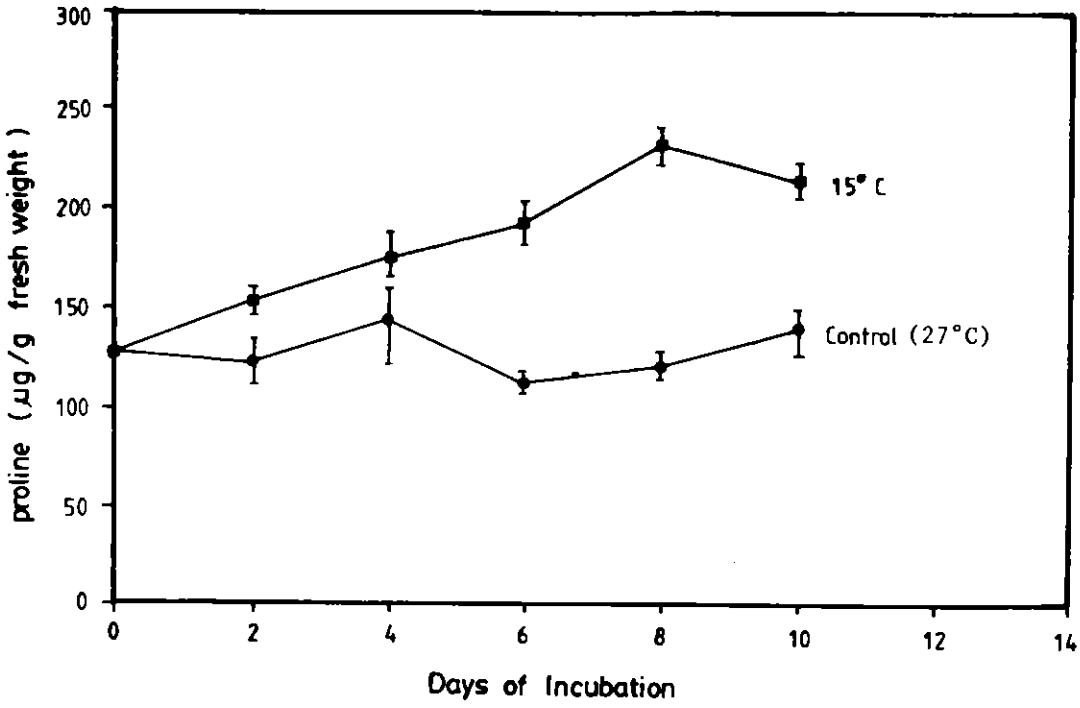


Figure 10. The effect of low temperature (15°C) on proline accumulation over 10 days in embryoid tissues. Each point represents the mean of three replicates ± S.E.

varieties were also found to increase by more than 30-fold (Lark and Dorffling, 1985). Teulieres *et al.* (1989) found that the proline content in the resistant lines of *Eucalyptus gunni* was higher when they were exposed to low temperatures.

In our study, accumulation of proline in oil palm PE cultures was about 2-fold higher than in the control. Kurshad and Yelenosky (1987) observed that the proline content was 3 to 4-fold higher in acclimatized citrus trees exposed to low temperatures. Van Swaaij *et al.* (1985) observed that the proline content increased by as much as 100 times in several genotypes of *Solanum* exposed to 2°C. Proline has also been reported to accumulate in herbaceous plants at the wintering stage (Sagisaka, 1987) and in wheat (Charest and Phan, 1990).

a. Control (0.1 M sucrose)
b. 10 mM proline
c. 0.5 M sucrose

Figure 11. The influence of proline and sucrose treatments on inducing low temperature tolerance in cultures (8 months old) at 15°C (1.5x)

Influence of sucrose and profile on temperature tolerance of PE cultures

Tarmizi *et al.* (1992) reported recently that 0.5 M sucrose and 10 mM proline added to PE cultures induced proline to accumulate at levels twelve- and forty-fold that of the controls, respectively. The sucrose treatment caused a reduction in moisture content, but the cultures appeared morphologically healthy. This level of sucrose, therefore, was used as an additional supplement for PE cultures maintained at low temperatures. It was observed that sucrose-treated cultures remained healthy when compared with the control for as long as eight to nine months under low temperature exposure conditions (*Figures 10* and *11*). This behaviour may have also been a result of proline accumulation, since the earlier study showed that PE cultures treated with 0.5 M sucrose at ambient temperatures, contained high levels of proline (Tarmizi *et al.*, 1992).

Oil palm polyembryogenic cultures were found to be sensitive to low temperature stress especially after a six-month exposure when tissue softening was noticeable (*Figure 12*). However, in the presence of sucrose this limitation was overcome. In several plant species it has been shown that drought or water stress can induce frost or low temperature tolerance (Cox and Levitt, 1969; Yelenosky 1979) and proline accumulation under these conditions has been related

a. Control (0.1 M sucrose)
b. 10 mM proline
c. 0.5 M sucrose

Figure 12. The influence of proline and sucrose treatments on inducing low temperature tolerance in cultures (8 months old) at 20°C (1.5x).

to stress tolerance (Van Swaaij *et al.*, 1985). As mentioned above, a high concentration of sucrose was found to induce proline accumulation and reduce the moisture content in oil palm PE

cultures (Tarmizi *et al.*, 1992). This shows that this treatment could be used in combination with low temperature storage. The physical appearance of the sucrose-treated cultures was better and they appeared to have a greater tolerance to extended low temperature exposures; they could be maintained for at least nine months, which was three months longer than for cultures subjected to low temperature storage alone. This treatment further prolongs subculture intervals and thus is suitable for medium term *in vitro* storage. Henshaw (1982) has proposed the use of reduced temperature in combination with increased osmolarity of the tissue culture medium, obtained by using sucrose up to 0.23M, for more effective storage of potato germplasm material. Bannier and Steponkus (1972) were successful earlier with sucrose supplements of 0.3 M and low temperature incubation for callus cultures of *Chrysanthemum morifolium*. Arora and Bhojwani (1989) also maximized the storage period of shoot cultures by combining low temperature treatment and altering the osmoticum.

The accumulation of proline induced by a high concentration of sucrose (moisture stress) (Tarmizi *et al.*, 1992) might be one of the added factors associated with the mechanism of low temperature tolerance in oil palm PE cultures. This suggestion is in agreement with Duncan and Widholm (1987) who reported that conditions which induce proline accumulation, including sucrose treatment, also increased cold tolerance of regenerable maize callus. This seems to imply that proline is beneficial during stress conditions and perhaps is an index of resistance, as suggested by Charest and Phan (1990) and Songstad *et al.* (1990). Several workers have also shown that plants that are tolerant to water stress are several degrees hardier than non-tolerant plants (Stout, 1980; Siminoritch and Cloutier, 1982).

CONCLUSIONS

The findings in this study indicate that low temperature treatments at 15°C and 20°C can be employed for minimal growth storage of *in vitro* cultures of oil palm for periods up to six months. The addition of 0.5M sucrose to the media as on osmoticum could further extend the storage period at low temperature to nine months. The application of low temperature regimes is a simple and practical procedure for prolonged storage of polyembryoids raised in tissue culture, if a reliable and dependable low temperature facility is available. An electrical power failure even for a short period of time will increase the incubation temperature and affect the morphology and physiology of the cultures. It can also induce the growth of contaminating microorganisms in the cultures.

Low temperature treatments induced proline accumulation in an oil palm clone tolerant of such treatments. The monitoring of proline content in various test clones may, therefore, provide an index to distinguish clones tolerant of exposure to low temperature regime from those susceptible to such conditions.

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