Journal of Oil Palm Research Vol. 12 No. 1, June 2000, p. 8-13

STUDIES TOWARDS UNDERSTANDING PROLINE ACCUMULATION IN OIL PALM *(Elaeis guineensis* Jacq.) POLYEMBRYOGENIC CULTURES

Keywords: Proline accumulation, oil palm polyembryogenic cultures, thioproline.

TARMIZI, A H* and MARZIAH, M**

* Malaysian Palm Oil Board, P.O. Box 10620, 50720 Kuala Lumpur, Malaysia.

** Faculty of Science and Environmental Studies. Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

Thioproline (a proline analog) at 10 mM induced proline (Pro) accumulation in oil palm polyembryogenic (PE) cultures. However, this treatment eventually killed the cultures, presumably due to some degree of toxicity. PE cultures utilized the exogenous proline (ExoPro) and ornithine (ExoOrn) more efficiently in liquid medium than in solid medium. The Pro accumulating *cultures which were treated with ExoPro, ExoOrn, or subjected to low temperature stress returned to normal levels on transfer to normal media and conditions. These reversible changes in cellular Pro concentration are consistent with Pro being readily utilizable and so it can be considered a labile metabolite in oil palm PE cultures.*

INTRODUCTION

Proline (Pro) in plant cells and tissues is a known physiological stress indicator as it accumulates under stress conditions (Aspinall and Paleg, 1981; Hasson and Poljakoff-Mayber, 1995; Gangopadhyay *et al.*, 1997). Pro also accumulated in oil palm PE cultures under moisture stress (Tarmizi *et al.*, 1992) and low temperature (Tarmizi and Marziah, 1995). In preliminary studies, Tarmizi *et al.* (1988) induced Pro production in oil palm PE cultures by treatment with abscisic acid. In order to further understand the accumulation of Pro in oil palm PE cultures, further studies were conducted. These included a study on the effects ofthioproline (T4C: a proline analog) and amino-oxyacetic acid (AOA: inhibitor of amino acid transaminases). In addition, the utilization of ExoPro and ExoOrn in the liquid and solid media were compared and the reversibility of level of accumulated Pro in oil palm PE cultures studied.

MATERIALS AND METHODS

PE cultures used for this study were taken from clone P10 and multiplied for the experiment. They were maintained on MS (Murashige and Skoog, 1962) medium (± 100 ml in 250 ml Erlenmeyer flasks), with slight modifications (Paranjothy et al., 1989) at 27°C-29°C with 12 hr photoperiod, 1000 lux light intensity (flourescent illumination, 90 μmol $m^{-2}s^{-1}$ PAR) and relative humidity at 60%-70%. The cultures were subcultured at 3-4 month intervals.

The Influence of Proline Analog and Enzyme Inhibitor on Pro Content in Oil Palm PE Cultures

Embryoids were cultured onto MS media supplemented with 10 mM thioproline (T4C), a proline analog, or 1 mM amino-oxyacetic acid (AOA), an enzyme inhibitor. The concentrations used were based on the effective concentrations reported by Elthon and Stewart (1984) on T4C and by Amrhein (1979) on AOA. The embryoids were analysed for Pro content at 10-day intervals for 30 days.

Efficiency of PE Cultures in Utilizing ExoPro and ExoOrn in Solid and Liquid Media

Embryoids were cultured onto MS media (solid or liquid media), supplemented with either 10 mM ExoPro and 10 mM ExoOrn. They were then analysed for Pro content at 10-day intervals for 30 days.

Time Taken for Pro to Return to the Normal Level in Treated Cultures

Oil palm embryoids were cultured onto MS media, supplemented with either 10 mM ExoOrn or 10 mM ExoPro, and incubated at 15°C to induce Pro accumulation. After 20 days culture [Pro was found to accumulate even after this time (Tarmizi,1992)], the embryoids were analysed for Pro content and some of the cultures transferred to MS medium and incubated at normal conditions (as described above). Pro content of the embryoids was analysed at various intervals to observe the pattern of Pro change.

Pro Determination

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

Statistical Analysis

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

RESULTS AND DISCUSSION

The Influence of T4C and AOA on Pro Content in Oil Palm PE Cultures

The effects of T4C and AOA on the Pro content in oil palm PE cultures were examined. T4C at 10 mM induced Pro accumulation to almost twice the level of the control, the highest being in the 10-day cultures (*Figure* I). However, the increment in T4C-treated cultures was low compared to the effect of ExoPro (Tarmizi, 1992). T4C produced some toxic effects on the cultures after one month (Figure 2).

A similar finding was also made in detached green barley leaves which showed that T4C inhibited Pro oxidation in turgid leaves thus increasing the Pro content of the leaves (Elthon and Stewart, 1984). Treatment of barley leaf sections with 0.1 mM T4C caused a marked

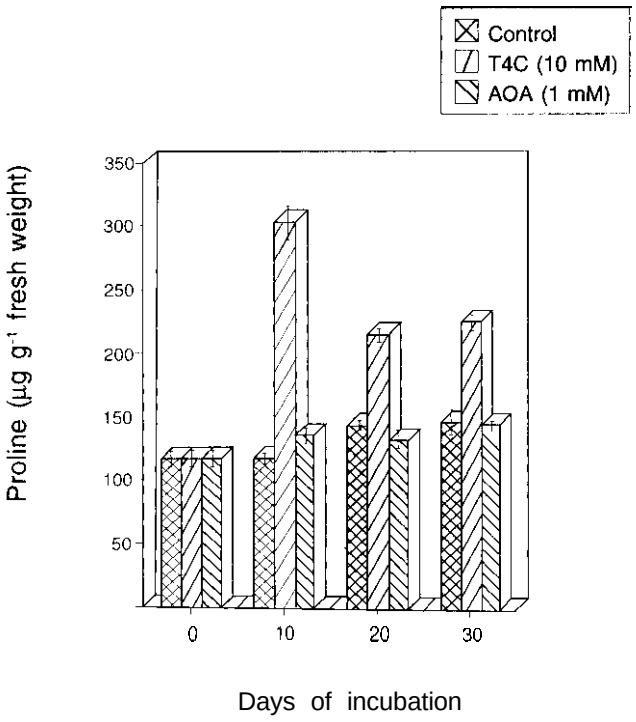

Figure I. The influence of thioproline and amino-oxyacetic acid on proline accumulation in the embryoid tissues. The data are a mean of three replicates ± S.E.

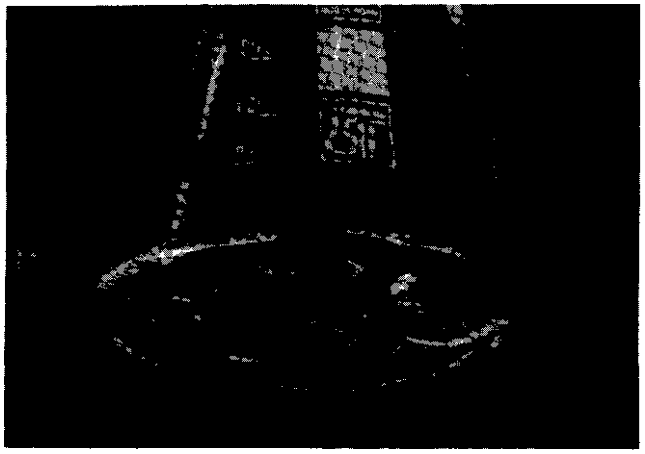

Figure 2. Thioproline at 10 mM induced proline accumulation and caused toxicity to the cultures (one month old: 1.1X).

increase in the Pro level (Beffagna *et* al., 1988). However, T4C at 10 mM, was toxic on oil palm polyembryogenic cultures inhibiting their growth and multiplication and eventually killing them. Currently, studies on the effects of different concentrations of T4C are in progress. ExoPro at high concentrations did not inhibit growth of the cultures (Tarmizi and Marziah, 1995). The inhibitory effect of T4C was reported by Kueh and Bright (1982) who found only three barley mutants resistant to this toxic effect. Other proline analogs also caused some toxic effects such as trans-4-hydroxyproline (Kueh and Bright, 1982) or azetidine-2-carboxylic acid (Vanlerberghe and Brown, 1987).

AOA is one of the enzyme inhibitors for aminotransferase (Dawsonet al., 1986). It affects the activity of ornithine aminotransferase which is involved in proline biosynthesis in oil palm PE cultures (Tarmizi, 1992). The effect of AOA on Pro accumulation in oil palm PE cultures was therefore studied. In oil palm PE cultures, the inhibitor did not significantly affect the proline content (Figure I). This could be due to the unspecific nature of the inhibitor as reported by John *et* al. (1978). A more specific inhibitor(s) could be used for oil palm cultures. The other reason for the lack of inhibition could be the unsuitable assay conditions for enzyme/substrate systems, which may need to be refined.

The Efficiency of PE Cultures in Using ExoPro and ExoOrn in Solid and Liquid Media

In a comparison between solid and liquid media, oil palm PE cultures utilized ExoOrn and ExoPro more efficiently in the latter (Figure 3). This resulted in accumulation of Pro in PE cultures. This information is useful for inducing low temperature tolerance in oil palm PE cultures. The uptake of ExoPro or ExoOrn in the liquid medium was more uniform as the cultures were in direct and constant contact with the medium. Gamborg (1982) reported that the growth rates of cell aggregates, cell clusters and single cells are generally higher in liquid medium because it provides better control of the growing condition for the cells which are surrounded by the medium. ExoPro was proven beneficial in plant tissue culture. Tao *et* al. (1997) reported that a simple culture medium of N6 mineral and MS vitamins supplemented with ExoPro was suitable for initiation and maintenance of cell suspension culture of rice. ExoPro was also useful as additional supplement to embryo initiation medium for pepper (Buyukalaca and Mavituna, 1996).

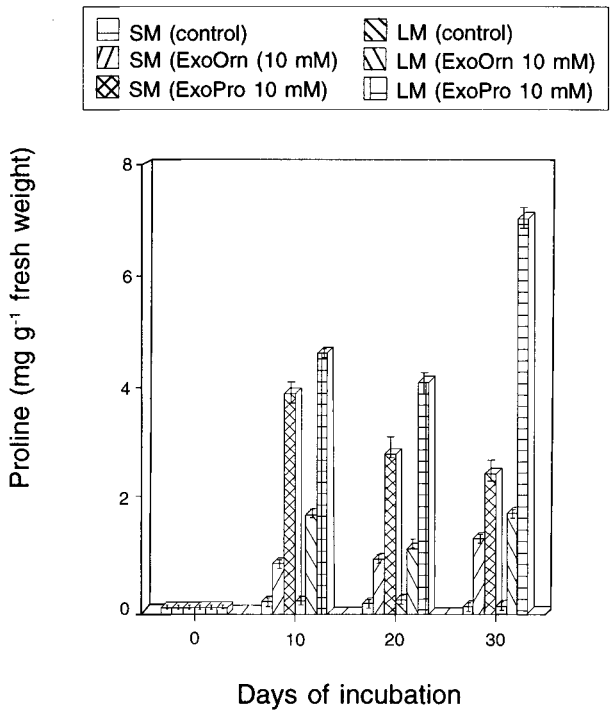

Figure 3. Comparison between solid (SM) and liquid media (LM) on proline accumulation in the embryoid tissues. The data are a mean of three replicates ± S.E.

Time Taken for Pro to Return to Normal Level in Treated Cultures

The Pro level accumulated in the cultures after combined treatments with ExoOrn, ExoPro and low temperature return to a normal when the cultures were transferred to normal conditions (*Figure 4*). Cultures treated with ExoPro and ExoOrn required about 40 days for full return to normality whereas the low temperature treated-culture required about 10 days. Wyn Jones and Storey (1978) reported a similar observation in which the Pro level in water stressed barley cultivars declined rapidly with rehydration. Handa *et al.* (1986) also observed decline in Pro level when water-stressed tomato cells were transferred back to the normal medium. The accumulation of intracellular Pro in response to water stress is not an injury response as the cells can revert back to normal growth upon removal of the stress. In this study, it was found that accumulated Pro was readily metabolized when transferred to normal growing conditions. Thus, Pro can be a labile metabolite in oil palm PE cultures. Alternatively, some Pro can be leached into the medium.

CONCLUSION

Studies on Pro accumulation suggested that T4C, a Proline analog, can induce Pro accumulation in oil palm PE cultures. However, toxic effects, observed after a month of treatment, eventually killed the cultures.

The utilization of ExoOrn and ExoPro which induced higher Pro accumulation in oil palm PE cultures, was more efficient in liquid me-

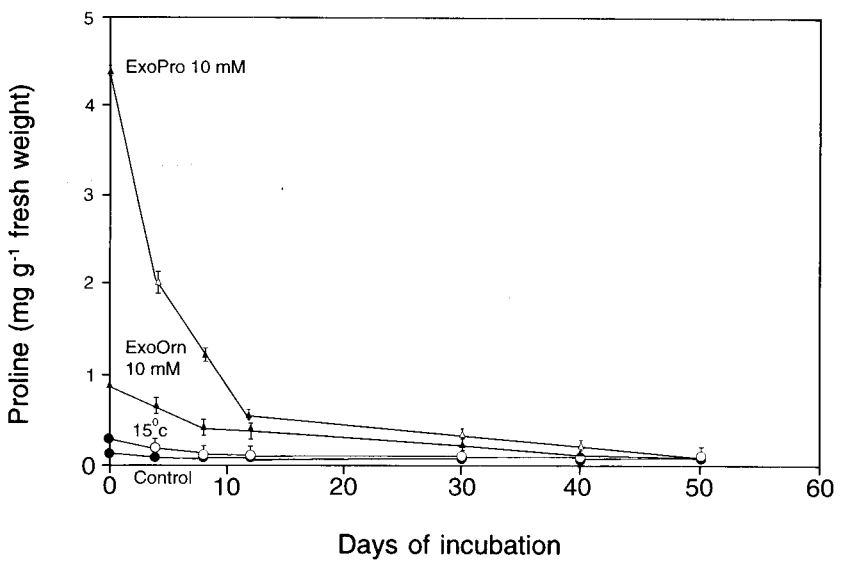

Figure 4. Reduction in proline content after transfer to normal conditions after 20 days of treatments. The data are a mean of three replicates ± S.E.

dium, and our preliminary studies on cryopreservation have shown that pretreatment of oil palm somatic embryos on MS solid medium supplemented with high concentrations of sucrose and proline can improve tolerance to liquid nitrogen freezing. These findings have indicated that liquid medium can replace solid medium in pretreatment of somatic embryos for cryopreservation.

The Pro accumulating cultures treated with ExoPro and ExoOrn or subjected to low temperature stress to return to normal levels on transferring to normal medium and conditions. This suggests that accumulated Pro is readily metabolized when transferred to normal conditions leading to restoration of a 'normal' cellular concentration. *This* being true, Pro can be considered a labile metabolite in oil palm PE cultures, although further investigations would be required to exclude other mechanisms, such as active extra-cellular transport, or leaching into the medium.

ACKNOWLEDGEMENTS

The authors wish to thank the Director-General of MPOB for permission to publish this paper. Thanks are also due to Dr Ariffin Darus, Dr Rajanaidu and Dr Douglas A Chamberlain for helpful comments on the manuscript. The technical assistance of Puan Fatimah is also gratefully acknowledged.

REFERENCES

AMRHEIN, N (1979). Biosynthesis of cyanidin in buckwheat hypocotyls. *Phytochemistry,* 18: 585-589.

ASPINALL, L D and PALEG, L G (1981). Proline accumulation: physiological aspects. In *Physiology and Biochemistry of Drought Resist*ance in *Plants* (Paleg, L G and Aspinall, D eds.). Academic Press, p. 205-241.

BATES, L S; WALDREN, R P and TEARE, I D (1973). Rapid determination of free proline in water stress studies. *Plant and Soil, 39: 205-*208.

BEFFAGNA, N; PESCI, P and RADICE, M (1988). Thioproline-induced accumulation of proline in barley leaf sections. *Plant, Cell and Environment,* 9: 141-144.

BUYUKALACA, S and MAVITUNA, F (1996). Somatic embryogenesis and plant regeneration of pepper in liquid media. *Plant Cell, Tissue and Organ Culture, 46: 227-235.*

DAWSON, R M C; ELLIOTT, D C; ELLIOT, W H and JONES, K M (1986). Other enzyme inhibitors. *Data for Biochemical Research.* Oxford Science Publication, New York. 317pp.

ELTHON, T E and STEWART, C R (1984). Effects of the proline analogue L-thiazolidine-4-carboxylic acid on proline metabolism. *Plant Physiology, 74:* 213-218.

GAMBORG, 0 L (1982). Callus and cell cultures. In *Plant Tissue Culture Methods* (Wetter, L R and Constabel, F eds.). Ottawa, *C.N.R.C.,* p. l-9.

GANGOPADHYAY, G, BASU, S; MUKHERJEE, B B and GUPTA, S (1997). Effects of salt and osmotic shocks on unadapted and adapted callus lines of tobacco. *Plant Cell, Tissue and Organ Culture, 49: 45-52.*

HANDA, S; HANDA, A K, HASEGAWA, D M and BRESSAN, R A (1986). Proline accumulation and the adaptation of cultured plant cells to water stress. *Plant Physiology, 80: 938-945.*

HASSON, E and POLJAKOFF-MAYBER, A (1995). Callus culture from hypocotyls of Kosteletzkya virginia (L) seedlings. Its growth, salt tolerance and response to abscisic acid. *Plant Cell, Tissue and Organ Culture, 43: 279-*285.

JOHN, R A, CHARTERIS, A and FOWLER, L J (1978). The reaction of amino-oxyacetate with pyridoxal phosphate-dependent enzymes. *Biochemistry Journal, 171:* 771-779.

KUEH, J S H and BRIGHT, S W J (1982). Biochemical and genetic analysis of three proline-accumulating barley mutants. *Plant Science Letter, 27:* 233-241.

MURASHIGE, T and SKOOG, F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15: 473-497.*

PARANJOTHY, K, ROHANI, 0; TARMIZI, A H; TAN, C S and TAN, C C (1989). Current status and strategies of oil palm research. In *Proceedings of the 1989 Palm Oil Development Conference*, 5-9 September 1989. Palm Oil Research Institute of Malaysia, Bangi. p. 109-125.

TAO, W J; LIU, B and XING, M (1997). Establishing Japonica rice suspensions retaining a high regeneration potential after 14 months of cultures. *Plant Cell, Tissue and Organ Culture,* 47: 213-216.

TARMIZI, A H; HANIFF, M H and PARANJOTHY, K (1988). Effect ofabscisic acid on growth and proline accumulation in oil palm polyembryogenic cultures. 7th FAOB Symposium, Kuala Lumpur. No. C-12.

TARMIZI, A H; MARZIAH, M and HALIM, A H (1992). Effects of various concentrations of sucrose on growth and proline accumulation in oil palm polyembryogenic cultures. In Agricultural Biotechnology: *Proceedings of Asia-Pacific Conference on Agricultural Biotechnology,* China Science and Technology Press, p. 461-464.

TARMIZI, A H (1992). Proline accumulation in oil palm polyembryogenic cultures. M.Sc, Universiti Putra Malaysia. 188pp.

TARMIZI, A H and MARZIAH, M (1995). The influence of low temperature treatment on growth and proline accumulation in polyembryogenic cultures of oil palm (*Elaeis* guineensis Jacq.). *Elaeis, 7(2):* 107-117.

VANLERBERGHE, G C and BROWN, L M (1987). Proline overproduction in cells of the green algae *Nannochloris Bacillaris* resistant to azetidine-2-Carboxylic Acid. *Plant Cell and Environment, 10:* 251-257.

WYN JONES, R G and STOREY, R (1978). Salt stress and comparative physiology in the gramineae. II. Glycinebetaine and proline accumulation in two salt and water-stressed barley cultivars. *Australian Journal of Plant Physiology,* 5: 817-829.