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Optimisation of regeneration systems for a range of Cassava (Manihot Esculenta Crantz) cultivars suitable for growth in South Africa and transformation with SACMV N-REP gene

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Chapter 4

SCREENING OF FOUR SELECTED SOUTH AFRICAN CASSAVA (*MANIHOT ESCULENTA* CRANTZ) CULTIVARS FOR PRODUCTION OF EMBRYOGENIC TISSUES

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4.1 INTRODUCTION

Cassava ranks second to sugarcane and is better than both maize and sorghum as an efficient producer of carbohydrate under optimal growing conditions (Fregene and Puonti-Kaerlas 2002). It is the most efficient producer under suboptimal conditions of uncertain rainfall, infertile soils and limited inputs encountered in the tropics (Loomis and Gerakis 1975; El-Sharkawy 1990).

However, biological constraints of a long growth cycle (8-24 months), vegetative propagation and perishability of the bulky roots lessen the crop's potential as an engine of rural development. The main attention in Africa has been on the viruses causing cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) (Hillocks and Thresh 2000). Securing sufficient clean planting material for the production of a healthy crop can be an ordeal for many small farmers and the relatively low inputs required for cassava production constraints sharply with the high inputs/risks involved in processing, transporting and marketing the highly perishable roots (Fresco 1993; Fregene and Puonti-Kaerlas 2002).

Biotechnology can contribute to the solution of these problems and realize great benefits for cassava farmers. Until recently, mainly due to selection problems, cassava was considered recalcitrant to genetic engineering. The first reports on successful regeneration of transgenic cassava plants have been published only in the second half of the 1990s (Li *et al*. 1996; Raemakers *et al*. 1996; Schöpke *et al*. 1996).

Somatic embryogenesis was first reported in cassava in 1982 by Stamp and Henshaw and since that time, the original embryogenic protocol has been improved upon an extended into several dozen cultivars (Szabados *et al*. 1987; Raemakers *et al*. 1993; Taylor *et al*. 1996). Of the different explants used for regeneration, meristems are the tissue of choice as they represent "growth centres" of plants (Fregene and Puonti-Kaerlas 2002). In cassava, meristems can be induced to form multiple shoots on cytokinin-containing medium. Most of the shoots are derived from pre-existing axillary meristems, but also *de novo* formation of new meristems and shoots occurs (Konan *et al*. 1997). Somatic embryogenesis is now the most commonly used regeneration method in cassava as it is mainly restricted to meristematic and embryonic tissues. Stamp and Henshaw (1982) induced somatic embryos on a limited number of explants such as cotyledons or embryonic axes from zygotic embryos, whereas (Matthews *et al*. 1993; Raemakers *et al*. 1993; Taylor *et al*. 1996; Puonti-Kaerlas *et al*. 1997; Li *et al*. 1998) induced somatic embryos from immature leaf lobes.

Primary somatic embryos can be induced to produce secondary somatic embryos by further subculturing on auxin-containing medium (Taylor *et al*. 1996). By constant subculturing of such embryos, a cyclic embryogenesis system can be established either in liquid or solid medium, where the embryos rarely pass the "torpedo" stage, until transferred to germination medium. The multicellular origin of cassava somatic embryos makes them poorly suited for genetic engineering (Fregene and Puonti-Kaerlas, 2002) as it results at best in the recovery of chimeric tissues.

Optimized conditions for generating embryogenic structures in different cassava cultivars are required if effective transformation systems are to be established for the crop. The transferability of both organogenesis-based regeneration and transformation capabilities to some African cassava cultivars has already been initiated at International Laboratory for Tropical Agricultural and Biotechnology (ILTAB), Missouri, USA by Taylor *et al*. (1996). These technologies must be expanded to regionally important varieties where cassava has an important socio-economic niche, like in southern Africa. This chapter reports on the research carried out at ILTAB which aimed to establish capabilities of four South African cultivars for embryogenic tissue generation.

4.2 MATERIALS AND METHODS

4.2.1 Culture conditions

Basal media were obtained from Sigma Chemical Company. Sucrose, growth regulators and vitamins were added before adjusting the pH to 5.8, addition of Oxoid agar and then autoclaving. Media were dispensed at 25 ml per Petri dish and cultured in a growth room at 25 ± 2 °C under a 16 hours photoperiod at 39 $\mu molm^{-2}s^{-1}$.

4.2.2 Plant material

Cassava cultivars from SA, namely T200 and T400 which are commercially grown by CSM, P4-4 and P4-10 which are also grown commercially for being high yielders, whereas TMS60444 and a Zimbabwean cv. TMS303337 were obtained at ILTAB, and were used for comparative purposes. All cassava cvs.

were maintained as shoot cultures in Murashige and Skoog (1962) basal medium supplemented with 2% sucrose (MS2). Mother plants were prepared by transferring nodal cuttings from the stock plants to MS2 at ten per Petri dish and subcultured at 4 week intervals.

4.2.3 Induction of embryogenic tissues

The induction of primary embryogenic tissue took place on MS2 supplemented with 50 µM picloram. *In vitro* immature leaf lobes were excised from mother plants and placed on MS2 supplemented with either 50 µM picloram alone or NAA (1, 10 and 25 µM). Further experiments with two SA-grown elite cvs. T200 and T400 were carried out under three light regimes [dark; low light (0.44 $\mu mol.m^{-2}.s^{-1}$) and high light (7.1 $\mu mol.m^{-2}.s^{-1}$)]. Fine forceps and a hypordemic needle were used to culture leaf explants. Leaf lobes were oriented with the abaxial surface in contact with the medium. Ten Petri dishes per treatment were used with each Petri dish containing ten leaf explants (hundred explants per treatment). After 28 days in culture, production of embryogenic tissues from leaf lobe explants was scored. A 0-5 range scale system developed by Taylor *et al*. (1996) was used where 0 denotes no embryogenic structures visible on leaf lobes whereas a scale of 5 denotes the entire leaf margin was full of embryogenic structures (Table 4.1). Embryogenic structures (clusters comprising of cells that are totipotential) were cut away from associated callus and subcultured on the same induction medium.

4.3.3 Regeneration of *in vitro* plantlets

Embryos were regenerated into plantlets by sequential subcultures on medium consisting of MS basal salts supplemented with 5 µM NAA and 2 µM benzyl aminopurine (BAP). Regenerated plantlets were grown and rooted on MS medium devoid of growth regulators prior to transfer to the greenhouse.

4.3 RESULTS AND DISCUSSION

A reproducible transformation procedure in cassava in developing countries necessitates the development of culture procedures that facilitate production of embryogenic tissues across a significant proportion of regionally important cultivars. In this manner, ILTAB offers training to third world scientists on the biotechnological tools required to solve cassava problems around the world. Taylor *et al*. (1996) developed a reproducible cassava genetic transformation protocol for the West African cassava cultivar TMS60444. This cultivar is considered an excellent model system due to its susceptibility to CMD and rapid storage root formation in the greenhouse.

The step considered to be the most important during the production of target tissues in cassava gene insertion is the induction of primary embryogenic tissues from the explant. Failure to establish efficient procedures for this step, in order to eventually produce transgenics through different systems, renders cassava genetic engineering impossible.

In this current study, organised embryogenic structures were successfully achieved from leaf lobe explants of all four SA cultivars, as well as TMS60444

and TMS303337. The frequency of somatic embryo production is summarized in Fig 4.1. SA cvs. T200 and T400 performed well, producing OES at frequencies (79 and 59%) approaching that of the model cv. TMS60444 (76%). Both cvs. T200 and T400 were shown to be significantly superior to other two SA cultivars P4-4 and P4-10 (23 and 51% respectively) and the Zimbabwean cv. TMS30333 (23%). Although cv. P4-10 scored a higher frequency of 51%, its embryo proliferation score was recorded to be 2, half of that of good cvs. TMS60444,T200 and T400 (Table 4.1) (Fig 4.2). This meant that cv. P4-10 proved its poor potential for embryogenesis as it would require more leaf lobes to produce desired quantities of OES.

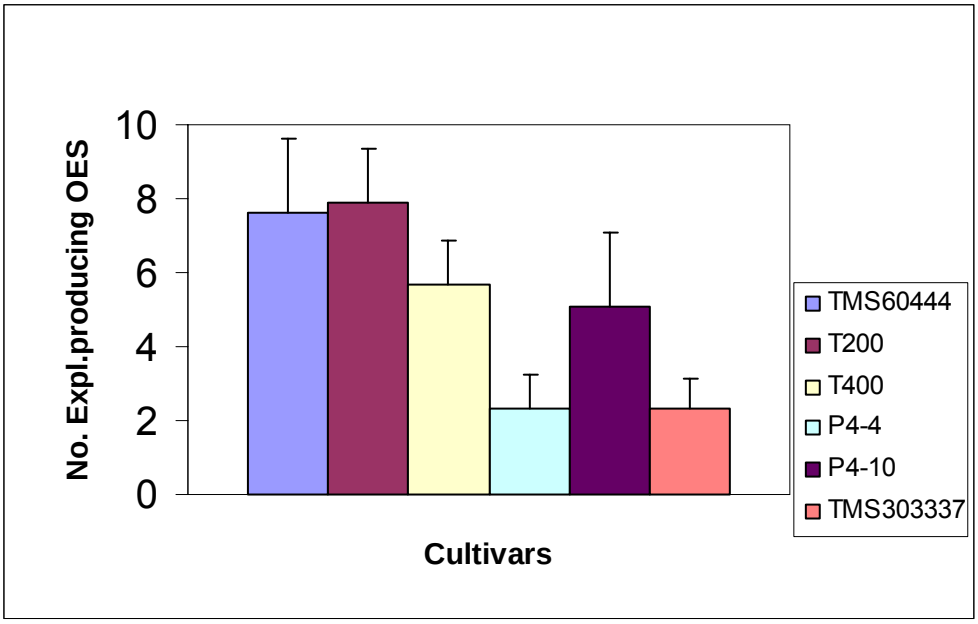

Fig 4.1 The effect of induction medium on the production of organised embryogenic structures from Southern African cassava cultivars. *Error bars* represent the standard error of the mean

Table 4.1 Amount of organised embryogenic structures produced by *in vitro* leaf lobe explants on induction medium[a]

Cultivar	Score
TMS60444	4
T200	4
T400	3
P4/4	1
P4/10	2
TMS303337	1

[a] Scores on a scale of 0 - 5 where 0= no embryogenic structures visible and 5 = most structures visible, entire leaf lobe (Taylor *et al*. 1996)

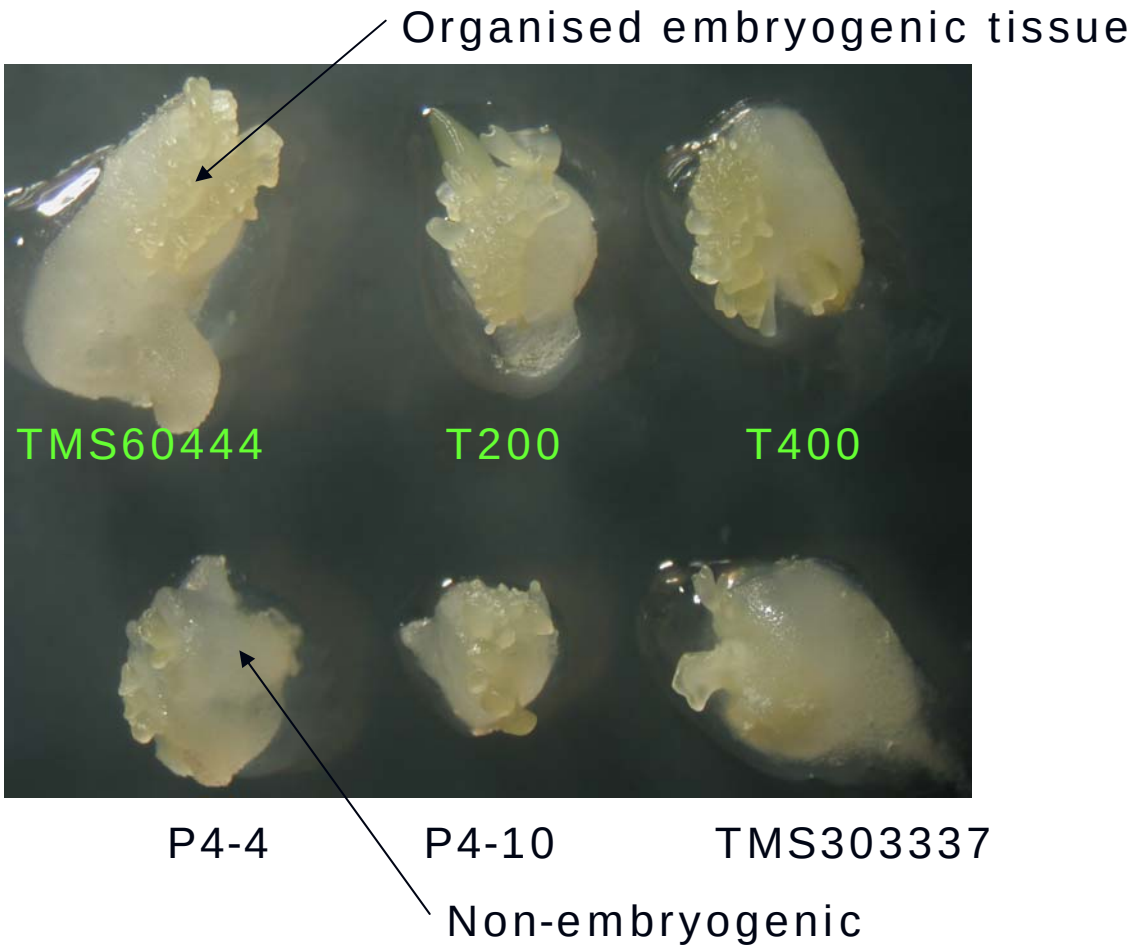

Fig 4.2 Organised embryogenic structures produced by immature leaf lobes after 28 days in MS2 medium supplemented with 50 µM picloram

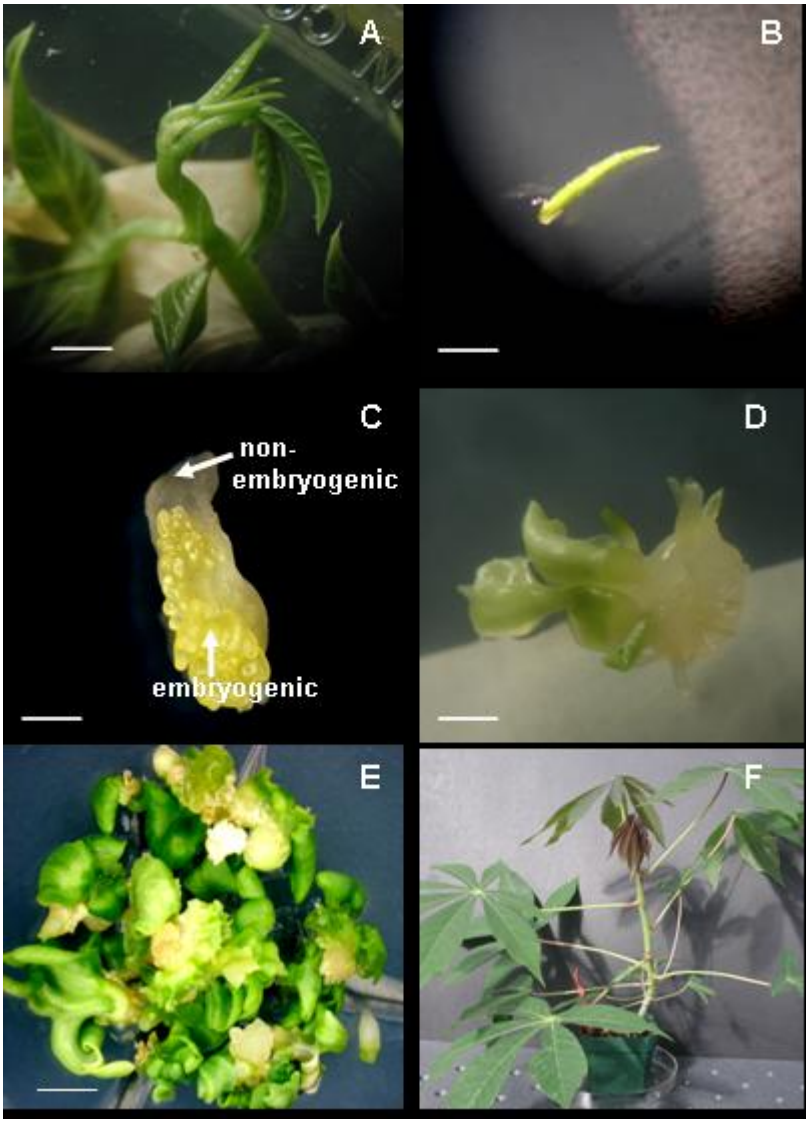

Fig 4.3 (A-F) Stages involved in OES production. (A) *in vitro* leaf lobes on the mother plant before excision, (B) a newly excised leaf lobe, (C) emergence of OES after two weeks surrounded by non- embryogenic callus (i), (D) mature embryos after four weeks of culture initiation, (E) multiple shoots four weeks on shooting medium, (F) 4 week old regenerated plantlets in greenhouse. bar = 1 mm

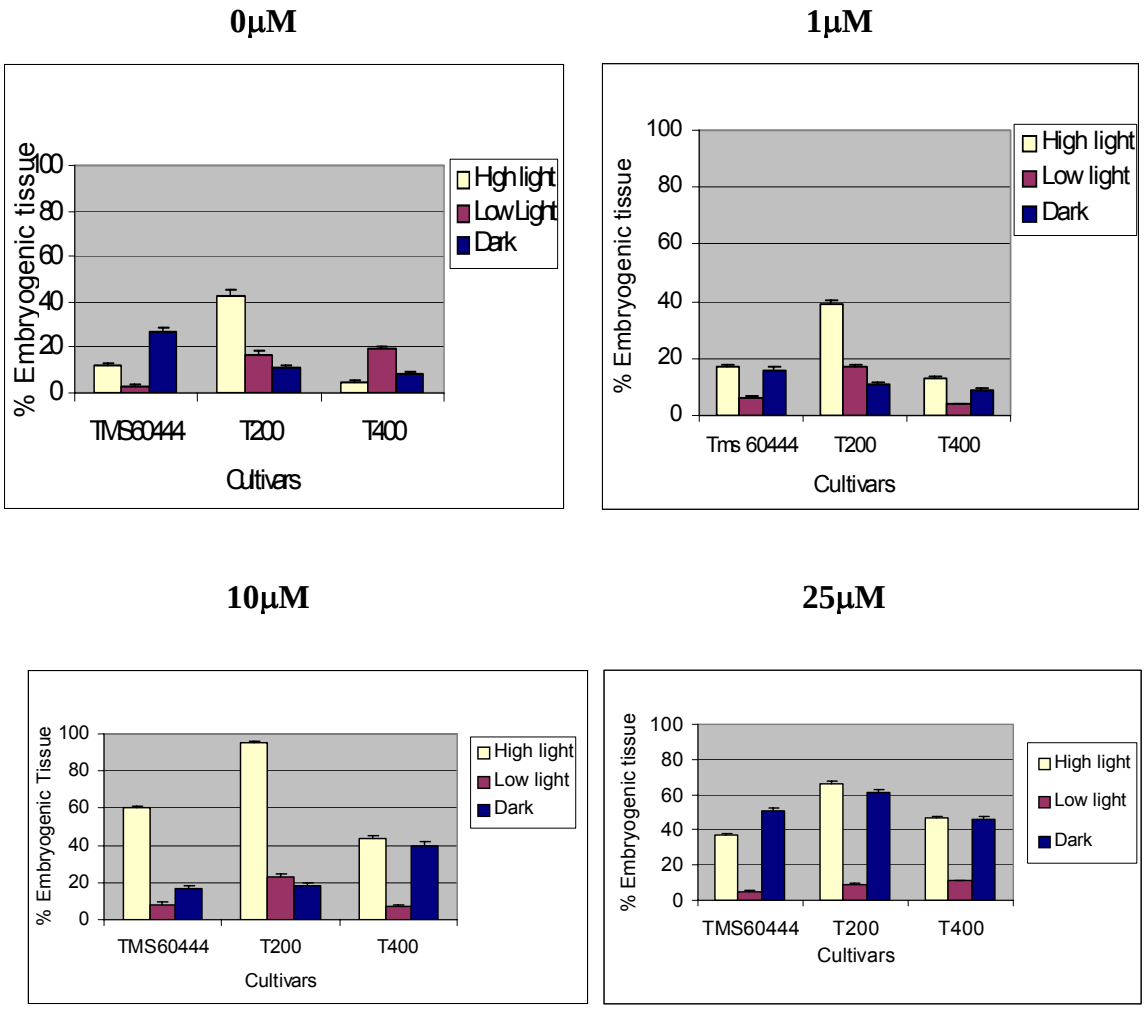

Fig 4.4 Influence of various NAA concentrations and different light regimes on the production of embryogenic structures in T200, T400 and TMS60444. *Error bars* represent the standard error of the mean

Taylor *et al*. (1996) also investigated the effect of including NAA (weak auxin) with picloram (strong auxin) on the production of embryogenic tissues. Concentrations of NAA ranging between 1 and 50 µM were added to MS2 medium containing 50 µM picloram. The results of their study indicated that the most effective concentration of NAA varied between 10 and 25 µM depending on the cultivar. The lowest and the highest levels attempted (1 and 50 µM) were the least effective with the inclusion of 50 µM NAA causing the induction frequency of embryogenic tissues to decline below that of the controls. The results of our study concurred with the findings of Taylor *et al.* (1996) by indicating that the two SA cvs. T200 and T400 plus the control cv. TMS60444 responded to various NAA concentrations (1, 10 and 25 µM) as well as NAA free media but differed significantly ($p<0.05$) between NAA concentrations (Fig 4.3). Addition of 10 µM NAA increased OES production in all cultivars. However, the addition of 25 µM NAA decreased OES production in cvs. TMS60444 and T200 but showed a slight increase in cv. T400.

In a study conducted by Akano *et al*. (2001), light levels were shown to have an important influence on the *in vitro* morphogenic potential of cassava tissues. Their results proved that low light (0.42 $\mu mol.m^{-2}.s^{-1}$) improved the formation of OES among the highly embryogenic cvs. With a Latin American cv. Tai-8 producing higher mass of OES than the model cv. TMS60444. Other cvs. like CM4574-7 (Latin American) and TME117 (African landrace) did not produce OES showing their poor potential for embryogenesis. Our current study also investigated different light regimes (dark, low light and high light) during OES

induction. In contrast to the results observed by Akano et al. (2001) under low light for cv. TMS60444, high light significantly improved OES production in the three cvs. TMS60444, T200 and T400 ($p<0.05$) (Fig 4.2).

Since both cvs. T200 and T400 were found to be excellent for the generation of OES, these cvs. were considered ideal for conversion into friable embryogenic callus (FEC), a preferred tissue for transgene insertion. The capability of producing FEC tissue from cassava cultivars also forms an alternative somatic embryogenic system. In order to generate FEC, OES produced (as described in materials and methods section) is cut away from any associated non-embryogenic callus and transferred to Gresshoff and Doy (1974) (GD) basal medium supplemented with 20 g/l sucrose and 50 µM picloram (Taylor et al. 1996). A study conducted by Kokora *et al*. (2001) showed that inclusion of tryptophan, which is a natural precursor of endogeneous IAA, to GD medium at 125 µM shortened the time required for the conversion of OES to FEC and increased the formation of FEC by 160% in cv. TMS60444. Findings of their study were extended to this current study in order to screen SA cultivars T200 and T400 for their potential to produce FEC. Various concentrations of tryptophan were included to test the beneficial effects of this amino acid on the conversion of OES. Both SA cvs. appeared to be responsive to this step with addition of 125 µM proving to be beneficial (Data not shown). Improvement was observed through the vigour of embryogenenic structures which were more compact and highly clustered (Fig 4.2) as opposed to structures induced in the absence of tryptophan that were scattered around the leaf lobe.

In order to regenerate plantlets *in vitro*, embryos of cvs. TMS60444, T200 and T400 were regenerated by sequential sub-cultures on media consisting of MS basal salts supplemented with 5 µM NAA and 2 µM BAP (Fig 4.3A-E). Regenerated plantlets were grown and rooted on MS medium devoid of growth regulators prior to transfer to the greenhouse. This was achieved by transferring tissues containing globular and torpedo embryos into regeneration media described earlier (MS with 5 µM NAA and 2 µM BAP). Cotyledon-stage embryos produced after four weeks were rooted and then after acclimatised into greenhouse conditions. Although data was not presented, embryos could be brought into maturity stage and regenerate with ease.

SA cv. T200 has proven to have optimum capability with T400 having poor capability in OES production. Findings of this study acknowledge the critical importance of all the above tissue culture stages and has recommended further studies that are currently underway in our laboratory in order to eventually optimize each one of them. We also conclude that light levels were observed to have important influence on the *in vitro* morphogenic potential of SA cultivars and therefore should be considered for different cultivars. Different SA cultivars or genotypes displayed varied response to induction of OES. Further screening of more SA cvs. for their potential to generate OES remains a top priority and also to optimize different tissue culture stages for each different cultivar.

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