DEVELOPMENT OF PLANT REGENERATION SYSTEMS IN AVOCADO *Persea americana* Mill.

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ABSTRACT

The first success in developing regeneration systems via somatic embryogenesis and shoot organogenesis in avocado (Persea americana Mill.) which are suitable for Phytophthora cinamomi resistance selection in vitro are reported.

Direct shoot regeneration was achieved from shoot tips or meristems excised from axillary buds cultured initially on MS medium supplemented with 0.5mg/L BAP and 0.1 mg/L NAA and subcultured on the same medium or with BAP in combination with 0.5 mg/L thidiazuron (TDZ). Increasing sucrose concentration of 70 g/L reduced the incidence of browning and improved shoot growth favoring the formation of multiple shoots.

Embryogenic calli were obtained from immature seeds of avocado cv RCF Purple cultured on modified Murashige and Skoog (MS) medium supplemented with 5.0 mg/L. 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/L 6-benzylaminopurine (BAP). Formation of somatic embryos was achieved among calli subcultured at 3-4 week interval for at least 4 cycles into either the same medium or with only 100 mg/L coconut water as additive. Various stages of somatic embryo development from the globular, heart-shaped to torpedo stages were observed.

Somatic embryos were subjected to maturation using MS-based medium containing 5 to 10 mg/L abscissic acid (ABA). Consequently, complete and efficient plant regeneration was obtained from mature embryos recultured in MS medium with 0.1 mg/L TDZ and 0.5 mg/L gibberellic acid (GA3).

Keywords: *avocado, Persea, somatic embryogenesis, organogenesis, tissue culture, plant regeneration.*

INTRODUCTION

Avocado (*Persea americana* Mill.) being the most nutritious of all fruits, has promising potential both in the local and export industry. Production in the

Philippines however, is limited to backyard planting and in addition, is beset by a fatal disease which also affects viable avocado industry in the United States of America and the South American countries. *Phytophthora cinnamomi* Rands causes root rot and attacks trees of all ages, including young nursery trees on tolerant rootstocks. The most obvious symptoms are gradual decline or dieback of foliage which are often wilted in appearance. Progressive defoliation occurs and entire branches dieback. Fruit production drops and eventually the entire tree dies (Coffey, 1991).

The use of resistant cultivars as rootstocks is so fai the most practical and durable disease control measure. However, most avocado cultivars are susceptible with the exception of 'Martin Grande' (Dolan, 1986). Other related species known to be resistant are either graft- or sexually-incompatible with avocado. Recent advances in cell and tissue culture enable the selection of resistance at the cellular level. This may potentially be applied in the selection of *Phytophthora*- avocado cell lines that subsequently grow into complete plants. For this strategy to work, two basic requirements must be met: a) an efficient plant regeneration system from cultured avocado cells or calli and b) the identification of an *in vitro* selection strategy.

This study was conducted to obtain basic information needed for the genetic improvement of avocados using biotechnology tools. Specifically, the objective was to develop a cell culture regeneration system of avocado.

MATERIALS AND METHODS

Direct Plantlet Formation

Plant Material. Healthy apical shoots were collected from greenhouse- and field-grown avocado cv RCF Purple during the dry and rainy season. With the leaves trimmed off and petioles intact, the shoots were washed with soap and water 5 times, followed by immersion in 70% (v/v) ethyl alcohol for 2 min and rinsed with sterile distilled water. This and all subsequent procedures were done inside the laminar flow hood under sterile conditions. Explants were soaked in varying concentrations of sodium hypochlorite (Zonrox, a.i. 5.25%) for 5-10 min followed by a rinse with sterile distilled water. Explants were cut into single nodes and again soaked in Zonrox and rinsed thoroughly in 5-8 changes of sterile distilled water. Meritems and axillary buds (0.5 mm) were excised from these materials and cultured on to shoot initiation medium.

Culture Media. Explants were cultured on three basal nutrients media, namely: Murashige and Skoog's (MS) (1962) with half-strength macro element components, standards MS medium and Schenk and Hidebrandt (SH) (1972) medium. All media were supplemented with 30g/L sucrose, 0.1 mg/L 1-Naphthalene Aceti Acid (NAA) and 0.5 mg/L BAP. Shoots formed *in vitro* were subcultured onto half strength MS medium with all possible combinations of TDZ (0.1 mg/L), GA_3 (0.5 mg/L), BAP (0.2 mg/L), sucrose (30 g/L) and agar (5 g/L) for shoot multiplication and rooting.

Culture Condition. The meristems/axillary buds were incubated in airconditioned room under diffused light approximately 10 μE m-2 sec-1 and photoperiod of 16 hr.

Callus Initiation, Somatic Embryogenesis and Plant Regeneration

Establishment of Embryogenic Cultures

Immature, 30 days old fruits of avocado cv RCF Purple were collected from the germplasm collection of the National Plant Germplasm Resources Laboratory, University of the Philippines Los Banos then rinsed in sterile distilled water. Under aseptic conditions, the immature seed was removed from the fruit and was dissected along its longitudinal and horizontal axes, thus producing eight explants. The seedpieces were placed onto sterile callus induction medium in test tubes so that the cut portion was in contact with the medium. The cultures were kept in complete darkness and subcultured onto fresh medium at 3 wk interval. The callus induction medium consisted of MS medium with half-strength macro components supplemented with 5.0 mg/L 2,4-D and 1.0 mg/L BAP.

Somatic Embryogenesis and Plant Regeneration

Somatic embryos on its globular stage formed in the same medium were transferred to similar medium with only 100 ml/L young coconut water as additive. Subsequently, somatic embryos on its cotyledonary stage were transferred to similar medium added with ABA at 5-10 mg/L. After 3 wk in culture, mature embryos were cultured onto MS medium with all possible combinations of TDZ (0.1 mg/L), BAP (0.2 mg/L), GA_3 (0.5 mg/L), sucrose (30g/L) and agar (5g/L) for germination.

RESULTS

Direct Plantlet Formation

The tissue culture requirements for initiating aseptic shoot tip/meristem and callus cultures were established in both field and greenhouse-grown avocado cv RCF Purple trees. Surface sterilization was achieved in most explants by soaking in 20% (v/v) Zonrox for 10 min and final washing in 10% (v/v) Zonrox for 5-8 min. Among field-collected materials, 60 and 100% asepsis was obtained during dry season (January to May) from axillary and meristem explants, respectively. During rainy season (June to November), per cent asepsis was lower and possible only through the use of smaller shoot tip/meristem explants. These results attested the failure of the technique which may be attributed to the change in weather conditions resulting to presence of some common contaminating organisms in field-grown trees, namely: *Aspergillus flavus*, *A. niger*, and *Collectotrichum* sp. However, these variations were not pronounced among leaf explants obtained from the greenhouse.

Shoot initiation was observed from intact axillary buds and exercised shoot tip/meristem cultured in modified MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA (Fig. 1). However, the ability of these cultures to develop directly onto shoots was greatly influenced by the source of explant. The excised shoot tip/meristem responded more efficiently (i.e., 100%) to shoot formation and did not exhibit callus formation as compared to axillary buds (Fig. 2). Subsequent transfers to media with varying concentrations of GA_3, and/or reduced concentrations of BAP and/or NAA resulted in futher shoot growth. When these shoots were transferred to medium containing 0.5 mg/L GA_3 and 0.1 mg/L TDZ, rapid shoot elongation and multiple shoot formation occurred 2 wk after transferring the shoots to medium. Subsequent transfer to MS basal medium induced root to form.

Callus Initiation, Somatic Embryogenesis and Plant Regeneration

Compact yellowish calli initiated from the lower half of the sectioned immature seeds of approximately 1 mo old avocado cv RCF Purple fruits cultured in MS medium with 2,4-D and BAP have regeneration potential (Fig. 3). The upper half of the sectioned immature seed germinated directly into shoot, and later ungerminated portion formed callus. Proliferation and maintenance was best achieved in MS medium with 5.0 mg/L 2,4-D and 0.5 mg/L BAP. Starting from the 5th monthly subculture, embryo-like structures formed in high frequency (Fig. 4). ABA at 5 and 10 mg/L added onto MS medium promoted maturation of somatic embryos while

Figure 1. Stages in shoot regeneration from avocado meristems.

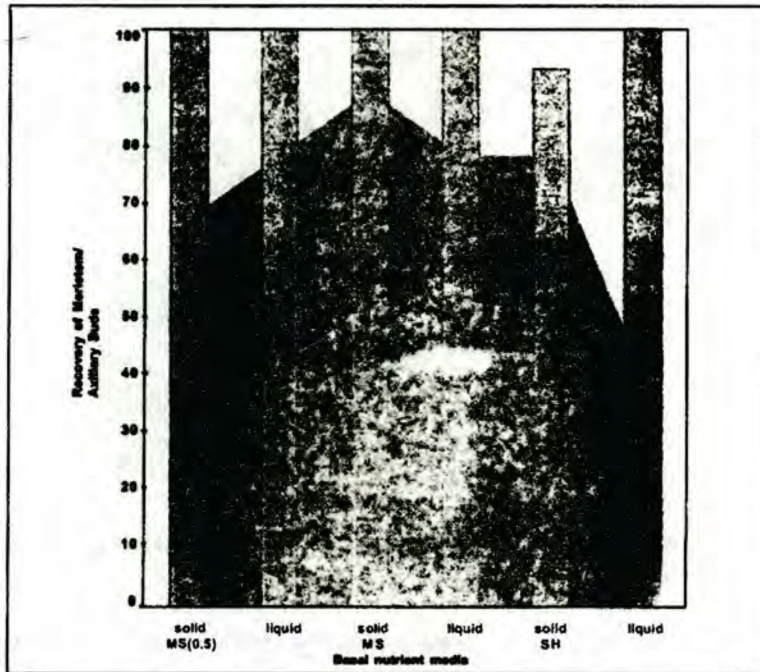

Figure 2. Effects of basal nutrients media on the recovery of responsive meristem/ axillary buds of field-grown avocado

Figure 3. Compact seed-derived calli of avocado with high regeneration potential.

Figure 4. Pro-embryoids and mature somatic embryos from seed-derived calli of avocado

mamitol at 15 and 30 g/L resulted to browning (Fig. 5). Results from 3 separate trials indicated that complete plant regeneration from seed-derived calli may be achieved following subculture of mature somatic embryos to MS medium with 0.1 mg/L TDZ and 0.5 mg/L GA_3, (Fig. 6).

DISCUSSION

The results demostrate that by manipulating media formulation, high frequency shoot regeneration can be achieved. From a single meristem/axillary bud, as many as 25 shoot buds was obtained in MS medium with TDZ and GA_3. Initiated meristem/axillary bud failed to elongate when subcultured to initiation medium but elongation and faster multiple shoot formation was achieved upon subculture to medium with 0.1 mg/L TDZ and 0.5 mg/L GA_3. The promotive effect of GA_3, on shoot bud elongation of avocado was evident, as was reported earlier in *Nicotiana* (Engelke, et al. 1973) and *Morus australis* (Pattnaik, et al. 1996). Root formation and elongation followed upon transfer to hormone-free MS medium with half-strength macro elements after 3 wk, however, the phenomenon of profuse growth of slender and thin roots was not observed.

Immature seed-derived calli having somatic embryogenesis potential may be induced to from proembryoids in medium containing auxin. While Picloram was cited as a critical source of auxin for somatic embryogenesis of avocado (Hammerschlag and Litz, 1992), findings show that 2,4-D at 5.0 mg/L in combination with BAP at 0.5 mg/L may be a good substitute for direct somatic embryogenesis. Likewise, secondary somatic embryogenesis from mature somatic embryos occurred

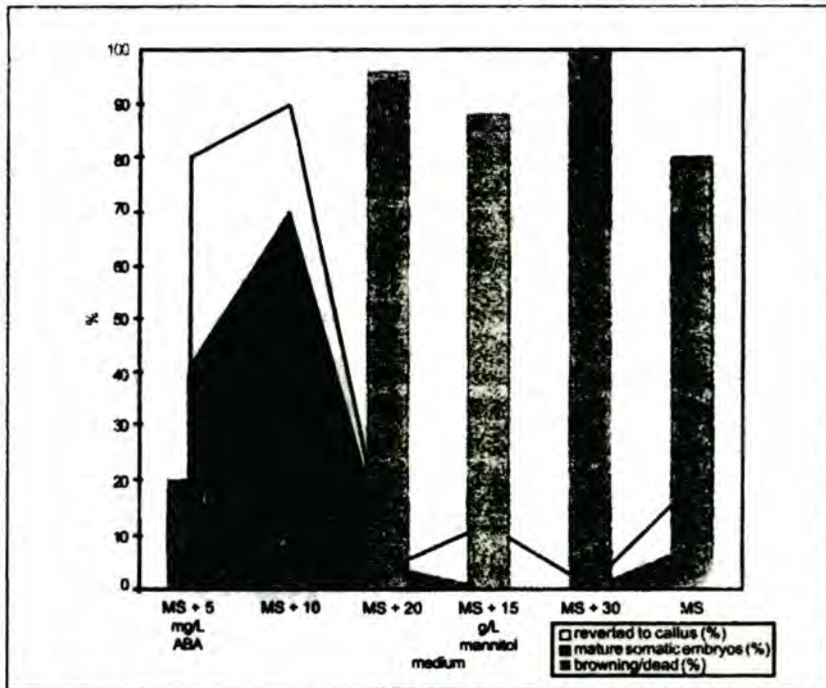

Figure 5. Effect if ABA and manitol on maturation of seed-derived somatic embryos.

Figure 6. Complete plantlet regenerated from avocado meristem (A) and seed-derived somatic embryos (B)

very efficiently in avocado, as in the case of *Vitis* (Krul and Worley, 1977; Gray, 1987) in medium with or without auxin. Somatic embryos subjected to maturation medium formed white cotyledonary embryos while browning of other portions not embryogenic was common. The browning may be the effect of the ABA used in maturation of somatic embryos which was effective in suppression of formation of accessory or secondary embryos from globular or early hearty-stage somatic embryos (Hammerschlag and Litz, 1992). The effect of GA_3, on germination of matured embryos of avocado was in congruence with its effect on *Citrus sinensis* (Kochba, 1974). The control in maturation and germination by ABA inhibition of precocious germination and GA_3, promotion of germination (Hammerschlag and Litz, 1992) was true for avocado seed-derived somatic embryos.

The regeneration systems for avocado are highly efficient and therefore suitable for in vitro selection strategy to select Phytophthora-resistance at the cellular level.

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