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RESCUE OF GENETICALLY TRANSFORMED AVOCADO BY MICROGRAFTING

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ABSTRACT

Development of avocado somatic embryos to maturity appears to be normal; however, the majority of somatic embryos lack bipolarity, often lacking a shoot apex. Developing shoots generally become necrotic in vitro. The germination and conversion rate of somatic embryos has therefore been low under optimal in vitro conditions. Avocado embryogenic cultures have been transformed with several gene constructs. In order to increase the plant recovery rate, shoots (5-10 mm long) that develop from somatic embryos have been grafted onto 3 to 4-week-old 'Booth' and 'Lula' in vitro-germinated seedling rootstocks with a success rate that is ca. 70%. The first nursery trials of transgenic avocado plants have been entirely derived from micrografted somatic embryo shoots.

Key Words: somatic embryo, avocado, micrografting

INTRODUCTION

Somatic embryogenesis of avocado has been described from zygotic embryos (Mooney and Van Staden, 1987; Pliego-Alfaro and Murashige, 1988; Raviv et al., 1998; Witjaksono and Litz, 1999a, b) and from the nucellus (Witjaksono et al., 1999a). Although somatic embryos can be recovered efficiently from embryogenic cultures, most of them are developmentally abnormal, and generally lack either a root or a shoot meristem (Pliego-Alfaro and Murashige, 1988; Witjaksono et al., 1999a; Efendi, 2003). The frequency of recovery of bipolar somatic embryos has been reported to range from 0.002% to 5-6% (Pliego-Alfaro and Murashige, 1988; Raviv et al., 1998; Witjaksono, 1997; Witjaksono and Litz, 1999a, b; Witjaksono et al., 1999a; Efendi, 2003), and is genotype-dependent.

Protoplast isolation and culture from embryogenic tissues of avocado has been reported (Witjaksono et al., 1998) together with limited somatic hybridization (Witjaksono, 1997). Embryogenic

cultures of avocado have been genetically transformed with selectable marker and reporter genes (Cruz-Hernandez et al., 1997) and with genes that target specific horticultural traits, including fruit ripening (Efendi, 2003) and various genes that are implicated in plant disease resistance, i.e., chitinase, β-1,6-glucanase and the antifungal protein gene (Raharjo et al., in press). Despite the progress that has been reported with respect to in vitro genetic manipulation of avocado, the low level of regeneration has impeded progress.

Witjaksono et al. (1999b) attempted to address this problem by micropropagating somatic embryo shoots; however, this procedure was not very effective, and many shoots that are dissected from somatic embryos fail to survive. Therefore, this strategy was inadequate because so much of the genetic diversity that was produced was lost. Another approach that has been attempted has been to improve the composition of the plant growth medium that is used for avocado somatic embryo development/maturation (Witjasono and Litz, 2002); however, the recovery of regenerants is unacceptably low for most manipulations. The current study describes another approach for efficient recovery of regenerants by micrografting somatic embryo shoots onto seedling rootstocks in vitro.

MATERIALS AND METHODS

embryogenic cultures

Embryogenic cultures were induced on $B5^-$ medium, i.e., B5 major salts (without NH_4NO_3) (Gamborg et al., 1968), MS (Murashige and Skoog, 1962) minor salts, 0.41 μM picloram and (in mg $liter^{-1}$) thiamine HCl (4), myo-inositol (100), sucrose (30,000) and agar (8,000) (Witjaksono and Litz, 1999a). Cultures were maintained on MSP, i.e., MS basal medium, with 0.41 mM picloram, the organic components of $B5^-$ and TC agar (8,000) (Witjaksono and Litz, 1999a). After 3 to 6 subcultures on MSP, embryogenic cultures were transferred into liquid MS3:1P medium, i.e., MS basal medium containing 60 mM inorganic nitrogen (75% of nitrogen is NO_3^- and 25% is NH_4^+) supplemented with organic addenda (Witjaksono and Litz, 1999b) and the other components of MSP.

Following their genetic manipulation in maintenance medium (see Litz, 2003 in this proceedings), suspension cultures were plated on somatic embryo development (SED) medium, i.e., MS medium supplemented with 20% (v/v) filter-sterilized coconut water, 45 g $litre^{-1}$ sucrose and solidified with 6 g $liter^{-1}$ gellan gum (Witjaksono and Litz, 1999b). The plates were closed but not sealed and were maintained in total darkness at 25°C for two months. Large, opaque-white (>5 mm diameter) somatic embryos were selected and germinated on semi solid MS medium containing 4.44 mM benzyladenine and 28.9 μM gibberellic acid, and incubated under light conditions consisting of 60-80 mmol $sec^{-1}m^{-2}$ provided by cool white fluorescent bulbs with a 16 h photoperiod. After 2-6 weeks, shoots emerged, and were used as scions when they were 5-20 mm length.

Petri dishes were sealed with Parafilm® and maintained in darkness at room temperature (25°C). Suspension cultures in Erlenmeyer flasks were sealed with aluminum foil and Parafilm, and incubated under ambient laboratory conditions at 125 rpm.

rootstock preparation

Mature avocado fruit were harvested from 'Booth' and 'Lula' trees in the avocado germplasm collection of the University of Florida TREC. The seeds were removed, and were surface-disinfested in a 20% (v/v) solution of commercial bleach for 10 min, followed by 3x rinses with sterile deionized water. The cotyledons were separated, and the embryo axis from each seed together with a cube of cotyledonary tissue (15x15x8 mm) was explanted onto semi solid MS medium in 200x25 mm

Petri dishes. The cultures were maintained under light conditions consisting of 60-80 μmol $sec^{-1}m^{-2}$ provided by cool white fluorescent bulbs with a 16 h photoperiod.

micrografting

A modification of a procedure for micrografting clonal avocado onto seedling rootstocks was adopted (Pliego Alfaro and Murashige, 1987). Each germinating zygotic embryo of 1.0-1.5 cm length was decapitated by a single slice through the hypocotyl approx. 5-7 mm below the cotyledonary nodes. A V-shaped cut, 3-4 mm deep was made with a new sterile razor blade. Excised shoot tips from somatic embryos (5-10 mm long) with a matching V-shaped base were inserted into the rootstock, and the graft was secured with sterile thread. Newly micrografted plantlets were transferred individually to semi solid MS medium with 100 mg $liter^{-1}$ activated charcoal in baby food jars, and were maintained under light conditions consisting of 60-80 mmol $sec^{-1}m^{-2}$ provided by cool white fluorescent bulbs with a 16 h photoperiod.

After 3-4 weeks, a successful graft union was established, and the thread that secured the rootstock and scion was removed. Vigorous growth of the grafted shoots generally required 4-15 weeks from the time that the graft union was established. With this procedure, 69.2% of micrografted transformed shoots developed normally and produced plants. In contrast, only 30.4% of shoots excised from somatic embryos (but not grafted) survived after 3 months, and surviving shoots failed to form roots and did not show normal shoot growth.

DISCUSSION AND CONCLUSIONS

Micrografting can be used to rescue genetically transformed avocados that develop from somatic embryos. This innovation is critical for the efficient recovery of genetically manipulated avocado, not only from transformed cultures but from cultures that are derived from other somatic cell genetic manipulations.

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