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High frequency somatic embryogenesis and plant regeneration from hypocotyl and leaf explants of gherkin (*Cucumis anguria* L.)

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ABSTRACT

Gherkin (*Cucumis anguria* L.) is an important commercial vegetable crop. An efficient protocol for plantlet regeneration from hypocotyl and leaf explants through direct somatic embryogenesis was developed. High frequency of somatic embryos (21.6 and 34.0) were obtained from hypocotyl and leaf explants when cultured on Murashige and Skoog (MS) salts plus B5 vitamins (MSB_5) supplemented with 5% sucrose, 1.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP and 150 μM L-glutamine. Initial culturing of embryos in the dark conditions for two weeks, followed by four weeks under light resulted in a higher frequency of embryo formation when compared to continuous light conditions. Histological observation showed that the somatic embryos originated from single cells of the epidermal layer. Histological evidence on formation of shoot and root poles during conversion of the embryos confirmed that these structures were true somatic embryos. Maturation of somatic embryos occurred on plant growth regulator-free MSB_5 semi-solid medium containing 3% sucrose and 0.5% (w/v) activated charcoal (AC). Conversion of embryos into plantlets was achieved on MSB_5 medium supplemented with 3% sucrose, 0.3% AC and 0.5 mg L^{-1} gibberellic acid (GA_3). Sucrose was found to be the best carbon source for SE induction, maturation and germination. Ninety percent of embryos were converted into normal plantlets. The plantlets were successfully acclimatized in the greenhouse with 95% survival rate and transferred to *ex vitro* conditions which developed with normal phenotypes. This regeneration protocol assured successful embryo induction and plantlet conversion. This is the first report for the induction of direct somatic embryogenesis from hypocotyl and leaf explants of *C. anguria*. The result of this study is beneficial for genetic transformation and mass clonal propagation.

1. Introduction

Gherkin (*Cucumis anguria* L.) is an important, highly nutrient vegetable and traditional medicinal plant, mainly cultivated and consumed in Africa, Brazil, Cuba, India, United States and Zimbabwe (Mangan et al., 2010). It is a member of the family Cucurbitaceae and genus *Cucumis*, which resemblance as a cucumber (Kirkbride, 1993). The crop is popularly known as 'pickling cucumber' or 'small cucumber' among the farmers (Nabard, 2005). The fruits of gherkin are consumed as boiled, fried, stewed, pickled, fresh in salads and also in hamburgers (Dzomba and Mupa, 2012; Matsumoto and Miyagi, 2012). In addition to its nutritional value, the fruits also contain high amounts of protein, calcium, phosphorous, potassium, iron and vitamin C (Whitaker and Davis, 1962). Moreover, the fruits, roots and seeds of gherkins are used in the traditional medicine, to treat stomach ache, jaundice, hemorrhoids and preventing stone formation in kidney (Baird and Thieret, 1988; Schultes, 1990). Phytochemists have isolated a number of potential medicinal components from *C. anguria*, such as cucurbitacin B, cucurbitacin D and cucurbitacin G (Sibanda and Chitate, 1990). Cucurbitacin B has potential to be used as a favorable phytochemical for cancer prevention (Promkan et al., 2013). *Cucumis anguria* consist of many useful compounds such as flavonoids, tannins, alkaloids, saponins and steroids, which contains high level

Abbreviations: AC, activated charcoal; 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; FAA, formaldehyde acetic acid alcohol; GA_3, gibberellic acid; IAA, indole-3-acetic acid; KIN, kinetin; NAA, α-napthaleneacetic acid; SE, somatic embryos.

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of antioxidant activity (Dzomba and Mupa, 2012). Anthraquinones and saponins were used for antibacterial and antifungal activity of *C. anguria* fruits against clinical pathogens (Senthil Kumar and Kamaraj, 2010). The pickled gherkins ensure worldwide demand and many food companies have started to develop opportunities for large scale production of gherkin. The climatic conditions favor the cultivation of gherkin in India and is mainly focused for export over worldwide (Mohapatra and Deepa, 2012). Gherkin has been introduced in India in the year 1989 for commercial production mainly for exports and its cultivation is driven largely through contract farming (Venu Prasad et al., 2013). Agri export zones (AEZ) have been created for cultivation of gherkin in South India, particularly in Karnataka, nearly 100,000 small and marginal farmers are involved in gherkin farming and the state produced 265,000 tons of gherkin in 50,000 ha of land in 2010–2011 (Venu Prasad et al., 2013).

Gherkin like most other melon species is susceptible to bacterial, fungal and viral diseases (Lebeda, 1984; Krishnareddy et al., 2003; Alvarez et al., 2005; Srinivasulu et al., 2010; Matsumoto et al., 2011; Matsumoto and Miyagi, 2012). Although new cultivars have been developed by cross breeding (Modolo and Costa, 2004), yet no cultivar has been developed with resistance to all these diseases (Matsumoto et al., 2012). Conventional breeding method of gherkin is difficult because of interspecific and intergeneric incompatibilities. Genetic engineering is an alternative way to improve the specific trait in plant breeding programs. Application of biotechnology in plant breeding programs requires efficient *in vitro* regeneration procedures. Somatic embryogenesis (SE) is a desirable, fast method of plant regeneration which may be induced *via* direct or indirect pathway. For direct somatic embryogenesis, the embryos were produced directly on the surface of organized tissue and this procedure efficiently reduces the time required for plant propagation, which may be beneficial to minimize culture-induced genetic changes (Sivanesan et al., 2011). Moreover, this technique is useful for artificial seed production (Cheruvathur et al., 2013), cryopreservation (Adu-Gyamfi and Wetten, 2012), genetic manipulations (Khan et al., 2010; Rajesh et al., 2013), and large scale propagation (Menéndez-Yuffa et al., 2010). Somatic embryos through suspension culture has been reported from leaves (Vengadesan et al., 2005), hypocotyl explants (Andrýsková et al., 2009) in *C. sativus* and cotyledons (Asad et al., 2010; Bairwa et al., 2012) in *C. melo*. Direct induction of somatic embryos has been reported from cotyledon explants of *C. melo* (Gray et al., 1993; Moradmand et al., 2011; Naderi et al., 2011). The main limitation of cucumber embryogenesis has, until now, been the high frequency of abnormal embryo formation combined with a low frequency of plant regeneration (Cade et al., 1990; Debeaujon and Branchard, 1993; Wróblewski et al., 1995; Pellinen et al., 1997). However, to the best of our knowledge, there are no reports on the induction of direct somatic embryos in gherkin. Therefore, we have established an efficient protocol for the regeneration of plantlets through direct somatic embryo induction from hypocotyls and leaf explants of *C. anguria* by studying the effect of growth regulators. The aim of the present investigation was to optimize the explants sources, growth regulators, environmental conditions, carbon sources and amino acids for the induction, maturation and germination of somatic embryos. We expect that our report would be helpful in the genetic transformation studies of *C. anguria*.

2. Materials and methods

2.1. Plant material

Seeds of gherkin (*Cucumis anguria* L.) were obtained from Nunhems Seeds Pvt., Ltd., Bangalore, India. The seeds were surface sterilized first with 70% (v/v) ethanol for 1 min and followed by 10 min treatment with 2% sodium hypochlorite (Sigma) and finally treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min. Then the seeds were rinsed with sterile water four times to remove the surface sterilant. Disinfected seeds were germinated on sterile moist cotton in dark for 48 h and the seedlings were grown under white fluorescent light (30 $\mu mol\,m^{-2}\,s^{-1}$) at a photoperiod of 16/8 h of light/dark and temperature 25 ± 2 °C. The leaves (10-mm^2) and hypocotyl (0.5-cm long) were used as explants from 7-day-old *in vitro* seedlings.

2.2. Effect of carbohydrates, amino acids and growth regulators for induction of somatic embryos

Leaf and hypocotyl explants were cultured on test tube (25 mm × 150 mm; Borosil, India) (one explant/tube) containing 20-mL aliquots of the embryo induction medium. The media containing MS salts (Murashige and Skoog, 1962) plus B5 (Gamborg et al., 1968) vitamins (MSB_5), different concentrations of carbohydrates [sucrose, glucose and maltose (3–6%, w/v)], amino acids [L-glutamine and L-asparagine (0–200 μM)] and supplemented with 0.5–2.0 $mg\,L^{-1}$ auxins [2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) and α-napthalene acetic acid (NAA)] either alone or in combination with 0.5–1.0 $mg\,L^{-1}$ cytokinins [6-benzylaminopurine (BAP) and kinetin (KIN)] and gellan gum (0.2%, Gelrite, Sigma). The medium was adjusted to pH 5.8 prior to autoclaving at 121 °C for 15 min. Explants were maintained for 0–14 days at 25 °C in darkness and then exposed to light of 30 $\mu mol\,m^{-2}\,s^{-1}$ with a light/dark cycle of 16/8 h. The experimental data were repeated twice and each treatment was performed with 10 explants in three replicates. The frequency of SE formation was determined by counting explants forming SE from the total number of the cultured explants after 40 days of culture. Explants were subcultured onto the same fresh medium at 15-day interval until the somatic embryos were induced.

2.3. Maturation of SE and conversion into plantlets

The SE were separated from the explants and cultured on glass test tube (25 mm × 150 mm; Borosil, India) for embryo maturation. The media consisting of MS salts, B5 vitamin without PGRs and 3% sucrose either alone or in combinations with activated charcoal (0.0–0.6%, w/v). The average number of cotyledonary stage embryos was counted using a stereo zoom microscope (MoticSMZ-143, Japan) attached to a computer. Torpedo and cotyledonary stage somatic embryos were transferred in the culture tube (25 mm × 150 mm; Borosil, India) onto MSB_5 media, 3% (w/v) sucrose supplemented and 0.3% (w/v) AC either alone or in combinations with 0.0–1.0 $mg\,L^{-1}$ GA_3 for germination and plantlet conversion. Somatic embryos were subcultured at intervals of two weeks. Gibberellic acid (GA_3) was filter sterilized and added to autoclaved medium. Other plant growth regulators were added to the basal medium prior to pH adjustment and sterilization. The embryo germination percentage was calculated as (number of germinated SE/total number of SE) × 100%. Only embryos showing both elongation of the root and development of a green shoot were considered to have germinated. In order to achieve further shoot and root development germinated plantlets were transferred to MSB_5 medium and incubated at 25 °C under cool white fluorescent light of 30 $\mu mol\,m^{-2}\,s^{-1}$ irradiance with a 16 h d^{-1} photoperiod for 4 weeks.

2.4. Acclimatization of plantlets

After germinated plants were removed from the culture tubes and washed in running tap water, they were transferred to plastic