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In vitro growth responses of two citrus species, i.e. Pummelo (*Citrus grandis* L.) and Key lime *(Citrus aurantifolia* Cristm.*)* in various sucrose concentrations

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Abstract

Studies were performed to investigate *in vitro* growth responses of seeds of two citrus species, viz. Pummelo and Key lime in various sucrose concentrations in an attempt to generate cost efficient *in vitro* citrus plant. Mature seeds collected from ripe fruits and after surface sterilization were cultured on different sucrose concentration containing MS media and compared them with control. The results were scrutinized by different parameters namely on seed swelling, radical and first root emergence, first and second pair leaf emergence. In seed swelling experiment, 2.50 % sucrose containing media for Pummelo and sucrose free medium for Key lime showed best performance. Radical emergence and first root initiation was observed best in 2.50 % sucrose containing MS media in both citrus species. First pair of leaf emergence was better in MS0 and 2.50 % of sucrose containing media in Pummelo whereas 1.0 %, 2.0 % and 2.50 % sucrose concentration showed better result in Key lime. In case of second pair leaf proliferation there were no impact of sucrose concentration on leaf formation but fast response depend on sucrose concentration and 2.50 % sucrose containing media showed best response in both citrus. Root induction from *in vitro* grown shoots-cutting was responded quickest in 1.0 % sucrose containing media in both citrus. Plantlets of both citrus species generated through *in vitro* culture were able to acclimatize successfully and confirmed natural growth on soil when transferred them in earthen pots.

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Introduction

Citrus is one of the most widely cultivated fruit in the globe and especially well known for their fine quality and unique savor. Pummelo (*Citrus grandis* L.) and Key lime (*Citrus aurantifolia* Cristm.) is member of Rutaceae family with genus *Citrus* (Hamza, 2013). Maximum citrus species and its hybrids are highly polyembryonic and are diploid (2n=2x=18) and also polyploidy, like triploid (2n=3x=27) and tetraploid (2n=4x=36). These are originated sexually during the fusion of non-reduced gametes or somatically by chromosome duplication in nuclear cells (Guerra *et al.*, 2014). Genetic relationship amongst citrus species showed that Key lime is a tri-hybrid intergeneric cross of Citron (*Citrus medica*), Pummelo, and a micro citrus species; *Citrus micrantha* (Carne, 1991). Whereas Pummelo is indicated as one of the true basic cultivated citrus species (Uzun and Yesiloglu, 2012). Pummelo and Key lime are polymorphic and highly heterozygous (Roose *et al.*, 1992). Usually Pummelo is 5-15 meter tall tree whereas Key lime is a small shrub like tree (Morton, 1987). Both of the citrus species execute better yields on freely draining soils and grow in acid to neutral soils with pH 5-8 but grow best at pH 6-7. Their growth rates are extremely patchy based on atmosphere, cultural practices, tree spacing, scion, and rootstock. They can tolerate a wide range of light, medium and heavy soil moisture but not excess soil salinity (Manner *et al.*, 2006).Citrus plants synthesize and accumulate in their cells a great variety of phytochemicals including low molecular phenolic, acetophenones, terpenoids, flavanoids, stilbenes and condensed tannins (Rajkumar *et al.*, 2014). These are most important citrus fruits among the other citrus species and act as a major source of citric acid and Vitamin C. They can be helpful for overcoming malnutrition problems related to Vitamin C which is very much wide spread in our country (Rahman *et al.*, 2003). *In vitro* growth response can provide basic data that can be implemented to overcome some constraints to citrus cultivation, quality improvement, resistance to disease and environmental stresses by means of conventional and transgenic technology. More over *in vitro* generated plantlets show more vigorous that can be used as explant for genetic transformation study and are commonly used as a source of ex plants for *Agrobacterium*-mediated transformation of citrus (Ananthakrjshnan *et al.*, 2007; Mendes *et al.*, 2002; Pena *et al.*, 1995; Yang *et al.*, 2000). Therefore present study was under taken to examine the effect of sucrose concentration for efficient growth response of seed of two citrus species; Pummelo and Key lime *in vitro*.

Materials and methods

Mature fruits of Pummelo and Key lime were peeled and the pulp removed to extract the seeds. Seeds from mature fruits were washed with running tap water and then treated with few drops of tween 20 to remove all pulp and adhering materials of seed surface. Thereafter, seeds were rinsed three times in distilled water. They were dipped for 3 minutes in 0.01 % $HgCl_2$ for surface sterilization with gentle stirring. The seeds were then rinsed seven times in sterile distilled water and cultured aseptically *in vitro*.

The basic nutrient medium consisted of MS (Murashige & Skoog, 1962) medium with vitamins and adjusted to pH 5.8 with 0.1 N NaOH and denoted as MS0 medium. Various concentration of sucrose i.e., 1.0 %, 2.0 %, 2.50 % and 3.0 % were added to MSO medium to make corresponding sucrose containing MS media without growth regulators. After adding 0.8 % Agar, the medium was sterilized by autoclaving at 121° C for 20 minutes, 105 kPa. Cultures were maintained at 22±2°C under 16 h photoperiod provided by cool white fluorescent light at photosynthetic photon flux density of 3000 lux. The culture tubes were checked regularly to record the response of the culture. Data were recorded on days required for seed swelling, days for first radical emerge, days for first root emerge, days for first pair leaf emerge, days for second pair leaf emerge and days for rooting from *in vitro* grown shoots.

Results

This experiment was carried out to identify the optimal concentrations of sucrose for *in vitro* seed germination and its subsequent growth followed by

root formation from *in vitro* grown shoots in Pummelo and Key lime.

Effect of different concentration of sucrose on in vitro swelling of seeds

Among treated sucrose concentrations Pummelo showed best response at 2.50 % sucrose containing media that had taken 3 days to optimal seed swelling and in other sucrose concentration needed more days to optimal seed swelling in Pummelo (Fig.1A and 1C). In case of Key lime it was observed that MS medium without any sucrose showed best media for optimal seed swelling response and it took 6 days to seed swelling. Addition of sucrose took more days in seed swelling in Key lime (Fig.1B and 1C).

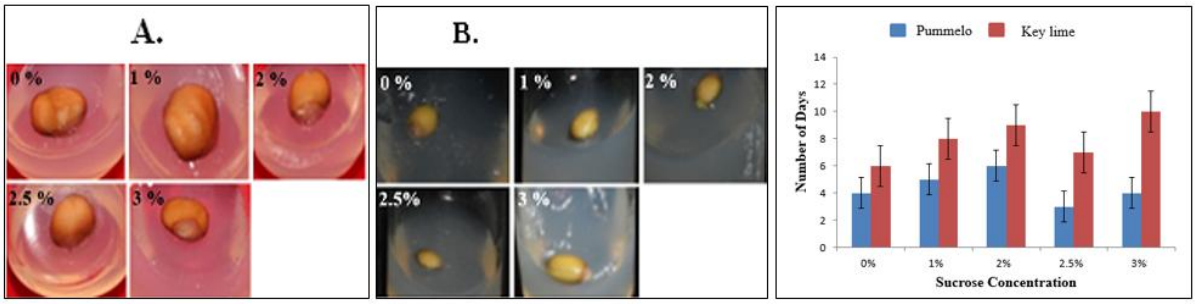

Fig. 1. Effect of different concentration of sucrose on *in vitro* swelling of seeds of two citrus species (A. Pummelo, B. Key lime).

Effect of different concentration of sucrose on in vitro first radical formation from seed

In case of radical formation it was found that both the citrus species showed the faster response at 2.50 % sucrose containing MS media and took 6 days and 9 days respectively. An extra addition of sucrose with this concentration halted radical growth both the citrus species. Among other media MS0 showed moderate response to radical emergence in both the citrus species (Fig. 2A, 2B and 2C).

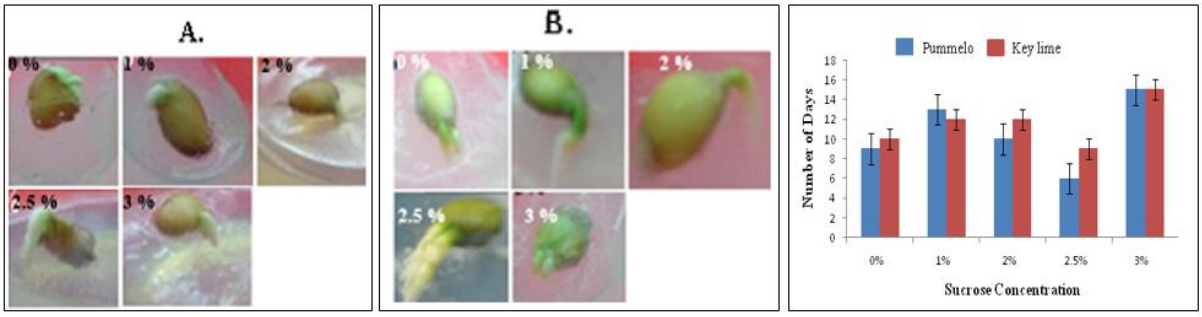

Fig. 2. Effect of different concentration of sucrose on *in vitro* first radical formation of two citrus species (A. Pummelo, B. Key lime).

Effect of different concentration of sucrose on in vitro root formation from seed

After touching emerging radical on the media data were recorded as root formation. Among various given sucrose concentration 2.50 % sucrose took least amount of days (14 days and 15 days respectively) for root formation in both citrus species whereas gradual decrease or increase of sucrose concentration reduced root growth (Fig. 3A, 3B and 3C). However 2.0 % sucrose containing media also responded fast in case of Key lime.

Effect of different concentration of sucrose on in vitro first pair leaf formation from seed

It was found that Pummelo citrus showed the faster response to first pair leaf formation in MS0 as well as 2.50 % sucrose containing media and took 22 days.

Other sucrose containing media took longer time to develop first pair leaf in Pummelo (Fig. 4A, 4C). On the other hand it was observed that addition of sucrose in MS0 media resulted faster first pair leaf formation but without sucrose as well as more sucrose than 2.50 % seized first pair leaf formation in Key lime (Fig. 4B, 4C).

Effect of different concentration of sucrose on second pair leaf formation from in vitro germinated seed

This part of experiment was conducted with the observation of second pair leaf formation from *in vitro* germinated seed in MS0 and various sucrose concentration containing MS media. Among different media combination 2.50 % sucrose containing media showed first response to develop second pair leaf from both citrus and took 28 days in Pummelo and 27 days in Key lime. Increase of sucrose concentration highly reduced second pair leaf development in Pummelo citrus (Fig. 5A, 5 B and 5C).

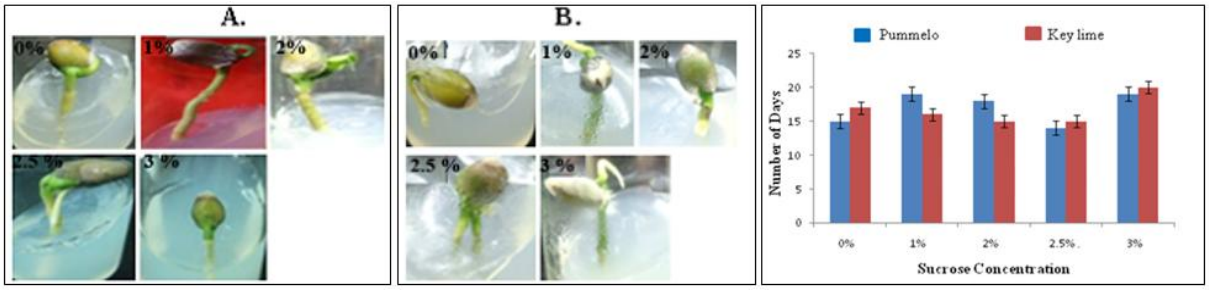

Fig. 3. Effect of different concentration of sucrose on *in vitro* root formation from seed of two citrus species (A. Pummelo, B. Key lime).

Effect of different concentration sucrose on in vitro rooting from shoots generated from in vitro germinated seed

In Vitro grown shoots were collected by scalpel blade when it reached 5 - 6cm long and cultured in different sucrose containing MS media to find out the most suitable concentration of sucrose for root formation.

Among different concentration of sucrose it was found that medium supplemented with 1.0 % sucrose showed quick response to induce root from *in vitro* grown shoots cuttings in both the citrus and MS0 as well as other sucrose containing media took longer time to induce root from *in vitro* grown shoots (Fig. 6A, 6C).

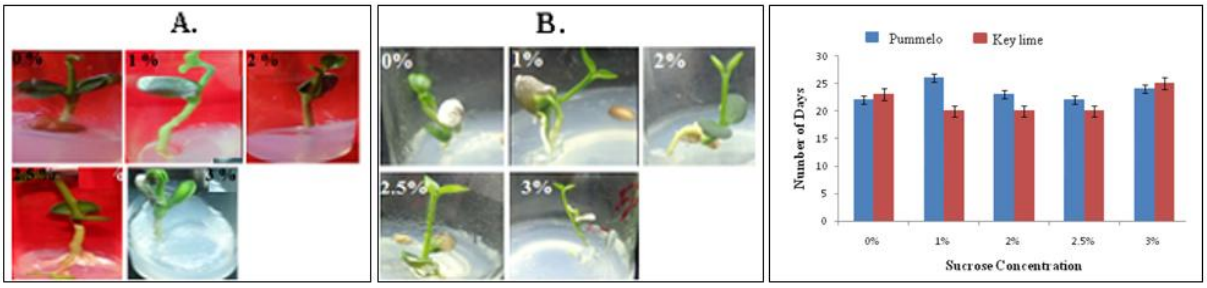

Fig. 4. Effect of different concentration of sucrose on *in vitro* first pair leaf formation form seed of two citrus species (A. Pummelo, B. Key lime).

Discussion

In vitro germinated seedling are commonly used as explant material for genetic transformation study in citrus as a common laboratory practice. In this study *in vitro* growth responses using hormone free medium supplemented with different concentration of sucrose were examined in order to get cost efficient citrus plantlets. All of treated media were capable of germination and root proliferation. However different growth responses were observed from different sucrose concentrations compare to control. In every parameter of observation *in vitro* response of

Pummelo and Key lime showed that growth response does not depend on sucrose concentration but fast responses depend on sucrose concentration and responded at a dose dependent manner. Related study in various crops observed similar results concluding that germination and seedling expansion was regulated by sucrose, and sucrose has additional complicated roles of faster response (Wu *et al.*, 2014).

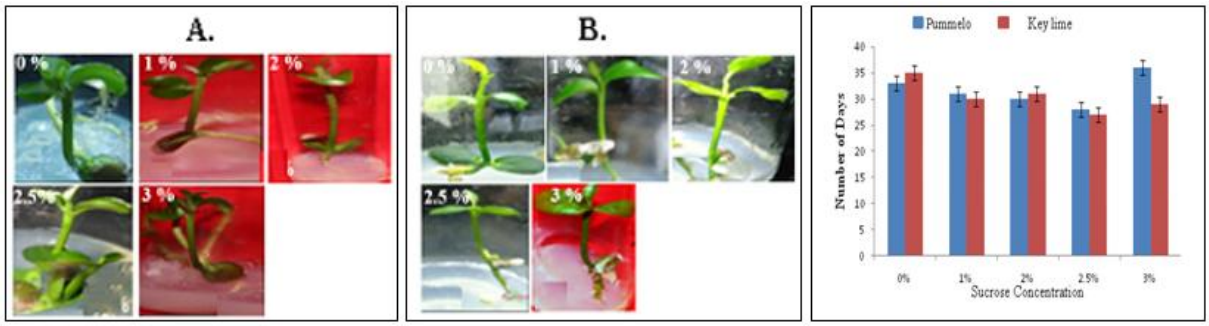

Fig. 5. Effect of different concentration of sucrose on second pair leaf formation from in *vitro* germinated seed (A. Pummelo, B. Key lime).

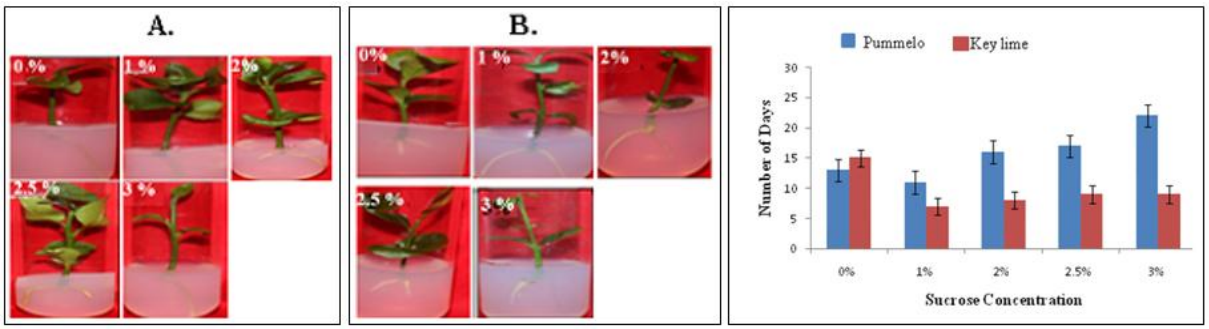

Fig. 6. Effect of different concentration of sucrose on *in vitro* rooting from shoots generated from *in vitro* germinated seed (A. Pummelo, B. Key lime).

There are four different sucrose concentration in addition to MS0 (without sucrose) were used in this study. During seed germination, seed swelling was observed in 6 days at sucrose free medium for Key lime and in 3 days at 2.50 % sucrose containing media for Pummelo. It was found that *in vitro* radical formation responses for both citrus species were quicker at 2.50 % sucrose. As mentioned earlier rooting data were collected when radical entered in the media and it was observed that *in vitro* root formation of Key lime was first at 2.0 % and 2.50 % of sucrose concentration whereas Pummelo was responded first at only 2.50 % of sucrose. This differences may occur due to the viability and vigor of seeds. It is noted that at similar sucrose concentration doesn't effect on root formation but fast response on root induction depends on sucrose concentration. MS medium supplemented with different concentrations of sucrose showed the differences of responses on *in vitro* first pair leaf initiation. Among these sucrose concentration, media containing 1.0 %, 2.0 % and 2.50 % of sucrose showed better responses for Key lime and 0.0 % and 2.50 % sucrose for Pummelo. However *in vitro* second pair leaf formation at 2.50 % of sucrose concentration showed the best responses for both citrus species. In case of second pair leaf formation it was found that there were no impact of sucrose concentration on leaf formation but fast responses depend on sucrose concentration. So it would be concluded that higher sucrose concentration had no effect on *in vitro* second pair leaf formation in the citrus species. According to the above analytic discussion it can be concluded that 2.50 % concentration of sucrose performed best in growth response during *in vitro* seeds germination of studied citrus species. Because 2.50 % sucrose concentration

showed the best positive result for radical formation, root formation, first leaf and second leaf initiation. However, root formation at 2.0 % sucrose and in first leaf formation at 1.0 % and 2.0 % was also found considerably good. These ambiguity of responses may occur due to the seed viability and other physiological factors regulating these stages. Shoots cutting about 5 - 6cm were collected from *in vitro* generated plantlets and subjected to root formation in various sucrose concentration (1.0 %, 2.0 %, 2.50 % and 3.0 % sucrose) and found that 1.0 % sucrose induce faster root than any other given concentration. It would also be noted that gradual increase of sucrose concentration proportional to gradual cessation off *in vitro* root formation from *in vitro* grown shoots-cutting for both citrus species. Similar findings also observed in other crops also where exogenous excess sucrose simply restrained the hypocotyls growth but encouraged root formation (Price, 2003 *et al.*, ; Xu *et al.*, 2010).From this surveillance it can concluded that, there is an impact of sucrose concentration on *in vitro* root formation in citrus. Lower concentration of sucrose showed faster growth rate of root where gradual increase of sucrose concentration causes sluggish speed of root formation. In case of acclimatization all plantlets showed positive response when these were transferred to soil and gradually naturalized to environmental condition.

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