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Table of Content: Volume 15 Number 32 10 August, 2016

ARTICLES

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Review

Propagation of *Gladiolus* corms and cormels: A review

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***Gladiolus* is an important estimated 8th in the world cut flower trade's cut flower grown throughout the world for its elegant attractive spikes of different hues and good keeping quality. The commercial cultivation of *Gladiolus* is based on natural multiplication of corms and cormels. However, multiplication rate of corms and cormels is slow and the conventional method of propagation is insufficient to meet the demand of planting material and eventually affect the final cost of corms. A number of improved conventional techniques including division of the corms, removal of leaf and flower spikes, use of standard corm size, and mechanical removal of sprouts can increase the multiplication rate of corms and cormels. These improved conventional methods of propagation are insufficient to meet the demand of planting materials. *In vitro* techniques are applicable for the propagation of corm producing species. These techniques are adopted at commercial level in order to fulfill supply gap of huge demand. A number of *in vitro* protocols have been developed for regeneration of *Gladiolus* plantlets using different media by using various explants sources of the plant. However, literature is rather scanty on *in vitro* cormel formation and acclimatization of *in vitro* propagules.**

Key words: Corms, cormels, galdiolus, propagation.

INTRODUCTION

Gladiolus is an important estimated 8th in the world cut flower trade grown for its elegant attractive spikes of different hues and good keeping quality (Sinha and Roy, 2002). The major producing countries are the United States (Florida and California), Holland, Italy, France, Poland, Bulgaria, Brazil, India, Australia and Israel. In the United States, the best-selling bulb is the *Gladiolus* with an estimated annual sale of more than 370 million corms (Narain, 2004). *Gladiolus*, a member of the Iris family with short life cycle of 110 to 120 days, require temperature regime between 10 and 25°C. *Gladiolus* comes under the category of bulbous plants. The bulbous plants are commercially perpetuated by using their underground storage organs such as rhizomes of tuberose, corms of *Gladiolus* and bulbs of lilies. However, there are other methods which are applied to these underground storage organs such as chipping, scooping, scaling, and scoring. These methods used for bulbs are not applicable for the propagation of corms as in *Gladiolus*. Unlike a bulb, which is predominantly fleshy leaf scales, a corm is a compressed solid thickened stem with distinct nodes and internodes (Hartman et al., 1990). Propagation of *Gladiolus* is principally by the natural multiplication of new corms and cormels (Hartman et al., 1990; Ziv and Lilien-

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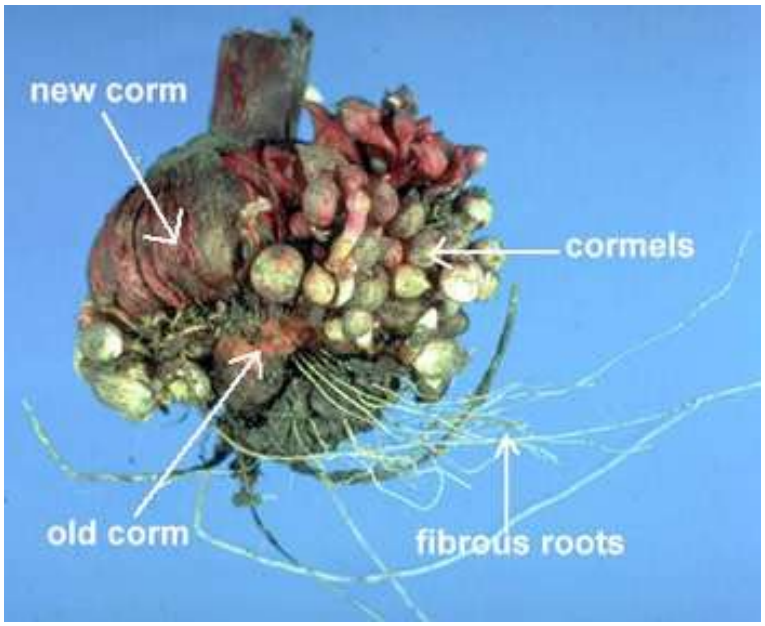

Figure 1. The *gladiolus* corm with cormels (http://montananaturalist.blogspot.com/2010/04/yellowbells-fritillaria-pudica.html).

Kipnis, 1990; Singh and Dohare, 1994; Bose et al., 2003). However, its commercial cultivation is limited by low rate of multiplication. One mother corm normally produces 1 to 2 daughter corms and about 25 cormels each season (Figure 1) (Misra, 1994; Sinha and Roy, 2002). However, daughter cormels developed from the axillary buds of the mother corm after one month of planting (Teixeira da Silva, 2003), require three to four seasons to attain standard size of flowering spike and daughter corms. The commercial production of corms and cormels is also greatly affected by *Fusarium* corm rot and high percentage of spoilage of corms during storage (Sinha and Roy, 2002; Riaz et al., 2010). This commercial production of corms and cormels does not fulfill the local demand of planting material and eventually affects corm cost. The dormancy of the corms and cormels is another problem in this regard (Priyakumari and Sheela, 2005).

Gladiolus commercial cultivation is not dependent on seed propagation as seed propagation is only used to evolve new and improved varieties by hybridization (Singh, 1992). Due to very low natural propagation rate, *Gladiolus* takes many years of growth before the cultivar can be released. Therefore, novel cultivars need to be rapidly mass multiplied by using *in vitro* propagation techniques in order to fulfill supply gap of huge demand of our local market which is not possible through conventional methods. These techniques increase multiplication rates (Novak and Petru, 1981; Takayama and Misawa, 1983; Wickremesinhe et al., 1994; Shabbir et al., 2009) and also generate material free from viruses and other pathogens (Blom-Barnhoorn and Van Aartrijk, 1985; Van Aartrijk et al., 1990). However, in many developing countries, the establishment cost of facilities and unit production cost of *in vitro* propagated plants is high, and often the return on investment is not in proportion to the potential economic advantages of the technology (Savangikar, 2004; Jo et al., 2008). This technology works in a way only when tissue culture methods are superior to conventional propagation, produce pathogen free plants in huge quantities, competition with conventional method and used for cloning.

A number of protocols have been developed on *in vitro* regeneration of plantlets in *Gladiolus*. However, literature is rather scanty on *in vitro* cormel formation. Since these pioneering efforts, a lot of data were generated and a number of papers have been published on different aspects of corm propagation and production in *Gladiolus*. A consolidated account of corm production techniques used in *Gladiolus* propagation is presented in this review.

PROPAGATION OF CORMS AND CORMELS THROUGH IMPROVED CONVENTIONAL METHODS

A number of improved techniques are used to promote corm and cormel production in *Gladiolus*. The research work was reported on divisions of the corms (Singh and Dohare, 1994; Memon et al., 2009a), removal of flower spikes (El-Gamassy and Sirry, 1967; Wilfret and Raulston, 1974; Mukhopadhyay and Das, 1977; Singh et

Figure 2. Sprouting on the basis of number of sprouts (Source: http://www.shutterstock.com/pic-3075215/stock-photo-one-gladiolus-flower-bulb-brightly-lit-isolated-on-white.html).

al., 1978; Misra, 1994; Singh and Dohare, 1994; Memon et al., 2009a), leaf clippings (Misra, 1994; Memon et al., 2009a), corm sizes (Farid Uddin et al., 2002; Memon et al., 2009b), manual removal of apical buds (Singh and Dohare, 1994) and mechanical removal of sprouts in succession during storage (Sharga and Basario, 1976; Misra, 1994). Each technique has its own merits and limitations to act as a satisfactory technique. The end users (growers of the *Gladiolus*) are unaware about these techniques. Very few and old references are available on these improved techniques used as conventional methods for the corm and cormel propagation of *Gladiolus*.

CONVENTIONAL IMPROVED TECHNIQUES

Corm divisions and sizes

The low rate production of corms and cormels is one of the major constrains in commercial cultivation of *Gladiolus*. Division of the corms in this regard is one of the best and economical alternatives to increase the yield of corms and cormels. Corm division is mainly based on the size of the mother corms and existing buds on the corm (Gromov, 1972). The size of the corms may be determined on the basis of minimum and maximum circumference or diameter. The North American *Gladiolus* Council (Wilfret, 1980) grouped corms into three grades on the basis of their circumference/diameter: Large, medium, and small. Jumbo (>5.1 cm) and No. 1 (>3.8 to ≤5.1 cm) categories come under the “large” category, whereas No. 2 (>3.2 to ≤3.8 cm) and No. 3 (>2.5 to ≤3.2 cm) are in the “medium” category. “Small” corms include No. 4 (>1.9 to ≤2.5 cm), No. 5 (>1.3 to ≤1.9 cm), and No. 6 (>1.0 to ≤1.3 cm). Circumference or diameter means the greatest dimension of the corm at right angles to a line running from the stem to the center of the basal portion.

Commercially, growers use whole corms of medium size (>2.5 to ≤ 3.8 cm) for getting the flower spike of standard size and daughter corms and cormels. However, when the objective is to get maximum production of corms and cormels, then it is better to use jumbo (>5.1 cm) and large size (>3.8 to ≤5.1 cm) corms. Commercial producers may be able to cut large corms instead of using whole corms for getting maximum corm and cormel production. Gromov (1972) reported that small corms are divided into 3 to 4 parts, large into 7 to 10 and very large ones may be divided into 12 to 15 parts depending on the number of the buds (Figure 2). Each division should have a bud and a portion of root zone. McKay et al. (1981) reported that division of large or number 3 corm sizes exhibited greater yield of new corms as compared with smaller size corms. They also reported greater inflorescence yield and higher inflorescence quality from large corms as compared with smaller corms. Lepez Oliveras et al. (1984) produced large number of grade one corms (4.8 cm diameter) in Peter Pears and White Goddes through division of corms planted in 50% peat and 50% perlite substrate, while soaking the corms for 24 h in 500 ppm GA_3 solution increased the cormel production. Gromov (1972) also reported that division of the corms markedly increased the growth of the filial corms, the weight of the corms, the

Figure 3. Memon et al. (2009a) used half corms of *gladiolus*.

number and weight of cormels in comparison with those produced from whole corms. If corm production is not the objective then medium sized corms are best to achieve an acceptable flower spike by market standards.

Singh and Dohare (1994) reported maximization of corm and cormel production in three cultivars (Pusa Suhagin, Mayur and Melody) of gadiolus using various improved cultural techniques. They obtained maximum number and weight of corms and cormels per plant in response to manual removal of two central apical buds. However, the reduction in weight and number of corms and cormels was observed in response to division to the half corms and quarter corms. When translated in terms of yield of corms per unit stock, plantation with quarter corms, showed maximum increase in yield over control (no improved cultural technique), followed by that with half corms. Memon et al. (2009a) almost obtained the same results by using half corms. They obtained half corms from whole corms with diameter of 3.6 to 3.8 cm (Figure 3). They used three varieties, namely, Traderhorn, White Friendship and Peter Pears and observed reduced yield of corms in each variety as compared to whole corms but yield of the corms was maximum on the basis of unit stock. On a unit stock basis, they observed increased yield of new corms 64% in Traderhorn, 36% in White Friendship and 37% in Peter Pears as compared to whole corms. They also produced jumbo size (>5.1 cm) corms from half corms as from whole corms.

Size of corm affects the vegetative, floral and corm yield attributes in *Gladiolus*. Smaller sizes of the corms are poor yielder, and larger sized corms add in cost of cultivation (Singh, 1992). Therefore, it is essential to find out optimum size of corms for obtaining the best results. Generally, it is advisable to have medium sized (> 2.5 to ≤ 3.8 cm) corms than small sized corms (> 1.3 to ≤ 2.5). Growers usually prefer small to medium sized corms for commercial cultivation of *Gladiolus*. The performance of large and medium corms was better with respect to corm and cormel production as compared with smaller ones (Mohanty et al., 1994). Similarly, other studies (Singh, 1996; Syamal et al., 1987; Kalasareddi et al., 1998) reported that large corms were superior in terms of number of shoots per corm, plant height, spike length, number of spikes, number of florets per spike and the diameter of corms produced. According to Hong et al. (1989), the number of daughter corms and flowering ability increased with increasing corm size up to 4 to 5 cm diameter, but there was no further increase for corms >5 cm diameter. In another study, Misra et al. (1985) studied the effect of 9 different corm sizes (from Jumbo to 0.6 cm in diameter) on flowering and corm production. They reported that the number of florets did not vary significantly up to 3.5 cm corm diameter. Number and weight of corms and cormels increased with the increase in corm size. These results are supported by the findings of Mukhopadhyay and Yadav (1984) who also reported more flowers, corm and cormel production from larger corms of 4.6 to 5.0 cm diameter. However, contradictory results were reported by Singh (1992) who reported production of more number of corms and cormels from large sized corms (6 to 8 cm diameter) than 5 cm.

Farid Uddin et al. (2002) studied the effect of corm size and depth of planting on the growth and flowering of *Gladiolus* cv. *Friendship* using the combination of four corm sizes (15, 10, 5 and 3 g) and three planting depths (10.0, 7.5 and 5.0 cm). Corm size had significant influence on all the parameters studied. Large corm (15 g) took

shortest time to complete 80% emergence (15.89 days) and flower initiation (60.44 days). Maximum plant height (97.56 cm), number of leaves (62.33), and length of flower stalk (26.07 cm) was observed from large sized corm planted at 5.0 cm depth and the lowest from very small corm (3 g) planted at 10 cm depth. Further, they observed that the plants planted with large sized corms showed the highest lodging but differed significantly with planting depth. The lodging was high in shallow planting (5 cm) than the deep planting (10 cm). Memon et al. (2009b) planted corms of three different sizes, namely, small (diameter 2.2 to 2.4 cm), medium (diameter 2.7 to 3.0 cm) and large (diameter 3.2 to 3.5 cm) from three different varieties of *Gladiolus*. They observed that large sized corms significantly increased the leaf breadth, length of flowering spike, and number of florets per spike over those produced from small and medium sized ones, whereas plant height was greatly decreased in response to large sized corms. Regarding corm production, large sized corms produced significantly higher weight of corms per plant, cormels per plant and combined total weight of corms and cormels per plant in all the three varieties of *Gladiolus*. However, variety Peter Pears produced the best results. The yield of new corms per plant was significantly increased in response to large sized corms both in White Friendship and Peter Pears, whereas, Traderhorn had no effect of corm size for number of corms per plant. Cormel production also depicted significant results in response to large sized corms in all the three varieties of *Gladiolus*. The results of Memon et al. (2009b) are in accordance with the results of Noor-ul-Amin et al. (2013). They planted cormels of white Friendship of three different sizes (>1.5 cm and < 2 cm, >1.0 cm and < 1.5 cm and >0.5 cm and < 1 cm) and observed the effect of various cormel sizes on the growth and development of gladiolus corms. They reported maximum percentage of sprouting (70.40) and survival (77.46) from large sized cormels. The greater number of leaves per plant (6.77), leaf area (61.14 cm^2), plant height (61.25 cm), diameter of corms (3.18 cm), number of cormels per plant (4.74) and corms weight (9.616) were recorded from large sized cormels. Kareem et al. (2013) also reported that large sized corms (3 to 3.5 cm) produced the best results in terms of vegetative growth and reproductive characteristics. More number of cormels per plant was also observed from large sized corms as compared to medium (2 to 2.5 cm) and small (1 to 1.5 cm) sized corms.

Clipping of leaves and flower spike

Gladiolus normally produces 6 to 7 leaves per plant, and depending on the variety, it may have 6 to 9 leaves (Misra, 1994). Misra (1994) reported critical leaf number per plant for proper corm and cormel growth is 4, and that the retention of 3 leaves per plant is sufficient for better corm growth if the spike is removed. The results of this study indicate that the removal of the flower spike and leaves (1 to 3) promote the development of corms and cormels in *Gladiolus* var. *Ratna's* Butterfly. This is because removal of few leaves conserves the plant's energy and metabolites that ultimately enhance the production of corms and cormels. However, if flower production is not the objective, the energy required for flower production may also be diverted towards corm and cormel development by removing the spike as well (Roberts and Milbrath, 1943; Halevy and Monselise, 1961; Mukhopadhyay and Das, 1977; Misra and Singh, 1979). Chowdhury et al. (1999) clipped off all seven leaves started from three to seven with or without flower spike. Better and significant results were found regarding corm diameter and weight of corms and cormel $plant^{-1}$ in response to removal of four leaves along with flower spike. Memon et al. (2009a) conducted a field experiment on the use of various improved techniques using three different varieties of *Gladiolus*. They used improved techniques included simple half corms (SHC) and half corms treated with activated charcoal (HCAC), clipping of three leaves (LR), and clipping of three leaves along with flower spike (LFsR). Whole corms (WC) were used as control. They observed the best response from the LFsR for number of cormels, number of corms and collective total weight of corms and cormels in each variety of the *Gladiolus*. On the basis of varietal comparison, White Friendship had more number of cormels (86.63) as compared to whole corms (71.57) (Figure 4). However, more collective total weight of corms and cormels was observed from Peter Pears (161.75 g) in response to LFsR as compared to WC (138.87 g) (Figure 5). Contradictory results were found by Ahmad and Siddique (2005). They found the best results in response to removal of only flower spike. They removed flower spike with one leaf, two and 3 leaves subsequently keeping control with no removal of spike and leaf. Removal of spike without leaf produced the highest number of corms (2.58), more weight (72.68 g), maximum diameter (6.19 cm) and volume (80.80 cm^3). Mukhopadhyay and Das (1977) also showed removal of spikes at early stages resulted in the increase of corm weight, whereas flower spikes removed along with two leaves had an adverse effect. Singh (1992) reported that corm yield increases by 60% when the flower spikes are removed as they appear, compared with plants on which the flower spikes are left to develop.

***IN VITRO* PROPAGATION OF CORMELS**

Mass propagation of cormels through modern technologies such as tissue culture techniques have been adopted at commercial level. Advanced countries are using highly sophisticated modern technologies for the commercial production of desired varieties in order to

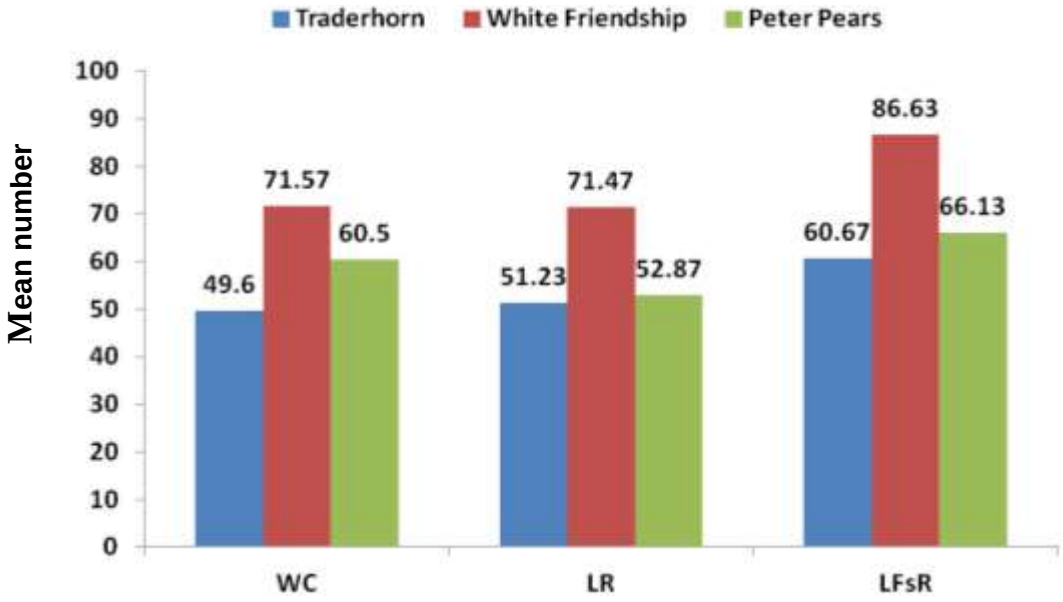

Figure 4. Mean number of cormels as affected by various improved cultural techniques in various varieties of *gladiolus* (WC = Whole corms; LR = clipping of 3 leaves; LFsR = clipping of 3 leaves plus flower spike). Source: Memon et al. (2009a).

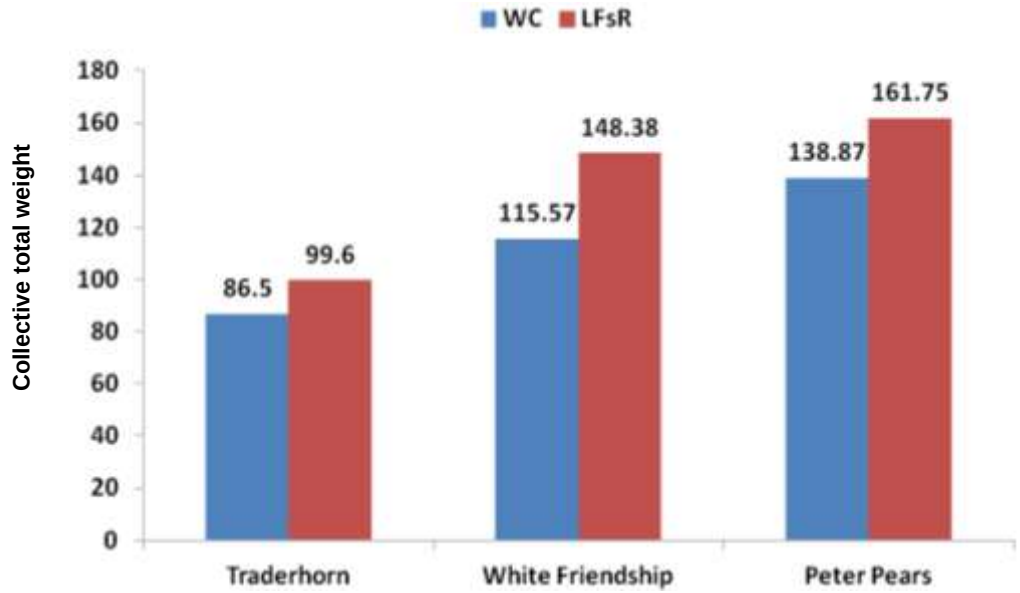

Figure 5. Collective total weight of corms and cormels as affected by clipping of leaves along with flower spike (LFsR) as compared to whole corms (WC) in various varieties of *gladiolus*. Source: Memon et al. (2009a).

compete in the international markets. This technology makes also possible to produce disease free and true to type planting material. *In vitro* techniques are useful for the propagation of corm producing species, because most of the hybrid cultivars of *Gladiolus* have a very low rate of multiplication. Since the pioneering efforts, a lot of data were generated and a number of papers have been published on different aspects of *in vitro* studies of *Gladiolus* with a greater emphasis on micropropagation. However, literature is rather scanty on *in vitro* cormel formation. A consolidated account of *in vitro* cormel propagation of *Gladiolus* is dealt with in the present

review.

In vitro regeneration of cormels

The ultimate goal of successful *in vitro* propagation of *Gladiolus* is the mass production of cormels (Steinitz et al., 1991; Dantu and Bhojwani, 1995; Sen and Sen, 1995; Al-Juboory et al., 1997; Nagaraju et al., 2002). The *in vitro* raised cormels can be easily stored and sown like seeds in plantation season (Wang and Hu, 1982; Ziv and Lilien-Kipnis, 1990). They may also reduce the transplantation difficulties which occurred during acclimatization (Ziv, 1979; Sengupta et al., 1984). Various explants such as nodal buds (Memon et al., 2010; Grewal et al., 1990; Arora et al., 1996), cormel tips (Arora et al., 1996), inflorescence stalk (Ziv et al., 1970), axillary buds of corm (Dantu and Bhojwani, 1987; Ahmad et al., 2000; Begum and Haddiuzaman, 1995) and slices of cormel sprouts (Sinha and Roy, 2002) have been reported to be used for *in vitro* cormel production in *Gladiolus* with the application of different growth hormones and sucrose in the medium.

GROWING MEDIUM REQUIREMENT FOR CORMEL FORMATION

The chemical composition of the growing medium is the most important factor for successful micropropagation and cormel development. Most of the reports of Gladiolus tissue culture indicated that Murashige and Skoog's (1962) medium supplemented with auxins and cytokinins is ideal for shoot initiation, multiplication and rooting (Lilien-Kipnis and Kochba, 1987; Logan and Zettler, 1985). However, addition of growth retardants and increased sucrose concentration improved cormel development (Ziv, 1989, 1990; Steinitz et al., 1991). Cormels can develop either using IBA or 2iP with different efficiency level, depending on the genotype; it is clear that in the presence of the cytokinin 2 iP either corms or shoots can develop from mother plant but in the presence of IBA the growth of shoots was strongly inhibited as reported by Ruffoni et al. (2012).

Sucrose requirement for cormel formation

Sucrose plays an important role for *in vitro* cormel formation in *Gladiolus* (Dantu and Bhojwani, 1987; Arora et al., 1996; Sinha and Roy, 2002; Memon et al., 2009b). It also has beneficial effect on multiplication of shoots (Kumar et al., 1999; De Bruyn and Ferreira, 1992), somatic embryogenesis (Loiseau et al., 1995) and rooting response of microshoots (Rahman et al., 1992; Romano et al., 1995). The increased growth of tuberous organs needs a relatively high (> 50 g/L) concentration of sucrose in the medium (Mares et al., 1985; Dantu and Bhojwani, 1987; Nagaraju et al., 2002). Higher concentration (6 or 10%) of sucrose favoured the formation of large corms (Dantu and Bhojwani, 1987). Hussain et al. (1995) reported that a high concentration of sucrose (5%) in combination with triadimefon resulted in 11 fold increase in size of in vitro corms in Cv. Friendship.

Most of the reports reported use of sucrose along with a rooting hormone such as indole butyric acid (IBA) or naphthalene acetic acid (NAA). Roy et al. (2006) compared agar-gelled medium with liquid medium supported with coir as the matrix at two different concentrations of sucrose (3 and 6%) by using basal portion of innermost leaves as an explant. They obtained large number of microcorms in liquid medium at higher concentration (6%) of sucrose as compared to agar-gelled medium. The addition of sucrose had a positive effect on *Gladiolus* culture weight, cormel number and weight in (Nagaraju et al., 2002). Other works (Ziv, 1979; Steinitz and Yahel, 1982; Sutter, 1986) reported that sucrose was totally utilized for corm filling as indicated by weight. Nagaraju et al. (2002) further reported that the plants grown in the presence of 12% sucrose in MS basal medium exhibited elongated leaves but small cormels. This suggests that sucrose is limiting growth in general and that the supply of carbohydrates from the leaves is not enough for cormel growth. According to Ziv (1979), the growth of these longer leaves was not related to the synthesis of more food by photosynthesis for the development of cormels. This might be due to the poor photosynthetic rate of *in vitro* cultures under low irradiance. Sinha and Roy (2002) produced three categories of corms, namely, small (5 to 10 mm), medium (10 to 15 mm) and large (16 to 22 mm) from rooted shoots cultured in half strength of MS supplemented with indole butyric acid (2 mg/L) and sucrose (6%). Memon et al. (2009b) obtained three different sizes of cormel production, namely, large (2.8 to 3.2 mm), medium (2.1 to 2.6 mm) and small (0.8 to 1.2) from rooted shoots cultured in MS medium supplemented with higher levels of sucrose (7%) but lower levels of IBA (1 mg/L) in variety White Friendship. Memon et al. (2014) observed the highest number of cromels (12.06) on MS medium supplemented with sucrose 5% plus IBA at 1 mg L^{-1} by using cormel slices of gladiolus (Figure 6) Jala (2013) cultured in vitro propagated propagules on MS medium supplemented with NAA 0.1 to 0.5 mg L^{-1} instead of IBA with sucrose (3%) got the highest number of cromels (5.8) per explant and fresh weight (144 mg per explant). De-Bruyn and Ferreira (1992) reported sucrose at 6 to 9% for *in vitro* cormel production. They also replaced sucrose by mannitol but could not find any beneficial effects on cormel production. Goo and Kim (1994) reported *in vitro* cormel formation from the shoot base of *Gladiolus* cv. *Topaz* was the greatest (90%) with 9% sucrose. Dantu and Bhojwani (1995) reported cormel formation from 96% of shoots on liquid MS medium supplemented with sucrose (6%). Kumar et al. (1999)

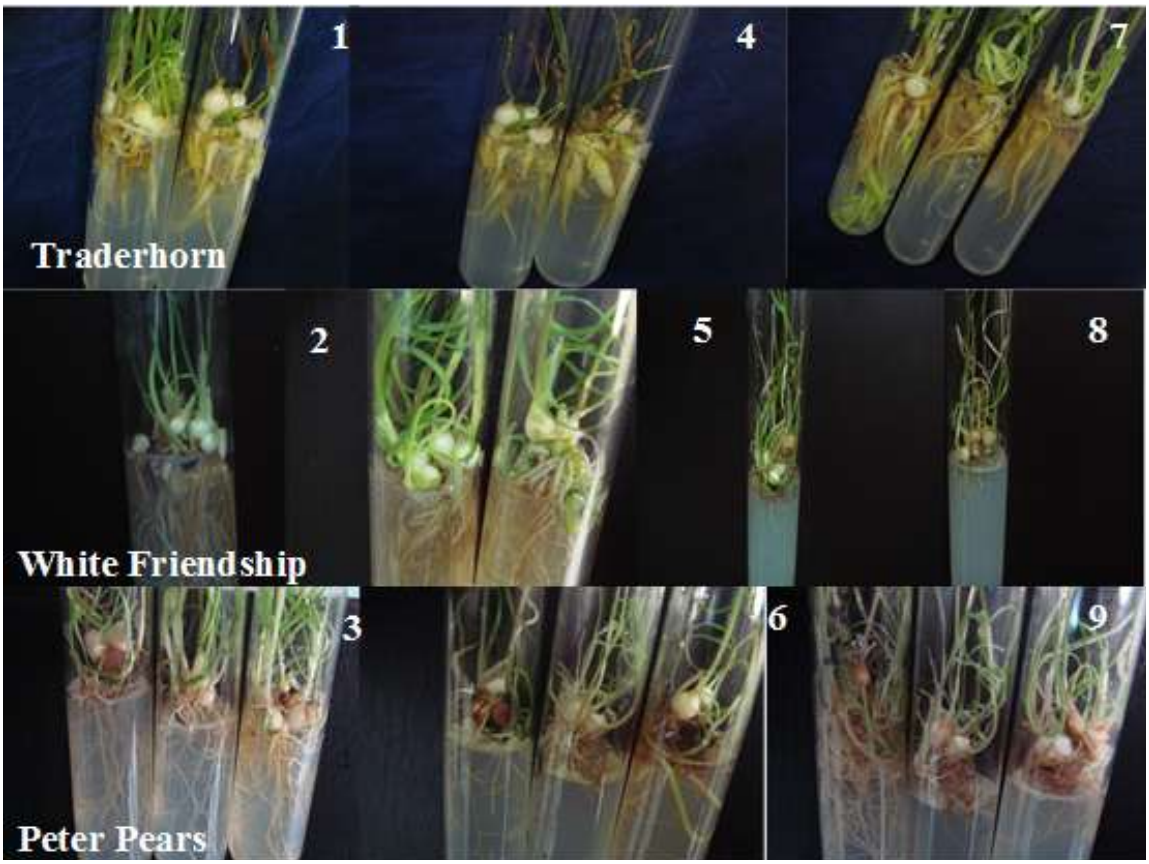

Figure 6. Cormel production from different explant sources. 1-3: Shoot tip of cormel on MS basal medium supplemented with IBA (1 mg L^{-1}) and sucrose (7%); 4-6: Middle and 7-9: Bottom slice of cormel on MS basal medium supplemented with IBA (1 mg L^{-1}) and sucrose (5%). Source: Memon (2010).

observed cormel formation on medium containing high sucrose concentration (> 6% and up to 12%).

Use of growth retardants for cormel formation

Growth retardants such as chloromequat (Kim and Han, 1993), paclobutrazol (Courduroux, 1967; El-Antalby et al., 1967), daminozide and ancymidol (Ziv, 1990) play major role in *in vitro* cormel formation in *Gladiolus*. Ziv (1990) produced cormels by using bud explants propagated in agitated liquid medium and supplemented with growth retardants like daminozide, ancymidol and paclobutrazol. The regeneration of buds was proliferated without leaves and these buds developed into procorms and after sub-culture to a hardening agar solidified medium, formed cormels 8 to 10 mm in diameter. Paclobutrazol with sucrose was also observed to be beneficial for *in vitro* cormel formation as reported by Nagarju et al. (2002). They observed large size cormels from the medium had paclobutrazol (10 mg/L) and sucrose (120 g/L) in MS medium. This report clears here that paclobutrazol (a retardant) with sucrose increased growth of the cormels by decreasing the growth of the leaves and stem. Reduction in stem elongation in several ornamental species was also reported by Coulston and Shearing (1985) due to the anti-gibberellin activity of paclobutrazol (Rademacher et al., 1984; Graebe, 1987) and promoted corm formation (Steinitz and Lilien-Kipnis, 1989; Ziv, 1989) when grown in media enriched with sucrose. Steinitz et al. (1991) reported that paclobutrazol using 10 mg/L, sucrose 60 g/L supplemented with BAP 3.0 mg/L promoted corm formation in liquid media.

Role of cytokinins in cormel formation

Very few reports were reported on the role of cytokinins in *in vitro* stimulation of tuberization (Palmer and Smith, 1970; Koda and Okazawa, 1983; Hussey and Stacey, 1984). There is apparent ambiguity about cytokinin role in the regulation of *Gladiolus* corm formation. Emek and Erdag (2007) reported corm formation on MS basal media contained Benzyl amino purine (BAP) at 0.1 mg/L. Kinetin induces cormel formation on excised stolon tips (Ginzburg and Ziv, 1973). BAP adversely affects corm

formation at the shoot base (Steinitz and Lilien-Kipnis, 1989). Ginzburg and Ziv (1973) used four plant hormones, namely, kinetin, gibberellin, abscisic acid and naphthalene acetic acid for cormel development in *Gladiolus*. Kinetin induced cormel formation, whereas, other three had no effect on tuberization. However, Kumar et al. (2002) observed corm formation on MS medium even without addition of growth regulators.

Acclimatization of *in vitro* propagules

Acclimatization of *in vitro* propagules to the *ex vitro* environment is a critical step for successful propagation. In *Gladiolus*, successful acclimatization can be possible by taking *in vitro* regenerants at three different stages: (i) When *in vitro* regenerated plantlets have optimum shoot/root ratio but no cormel formation; (ii) After cormel formation but before dormancy of the cormels; (iii) When cormels goes under dormant period and plant shoot dries up. Generally, the first option is in more practice in which *in vitro* regenerated shoots are planted into rooting medium and then placed into high humidity environment with low irradiance and temperature for acclimatization. It is necessary because (i) *in vitro* plantlets are not autotrophic (McCartan et al., 2004); (ii) poor development of leaf cuticle; and (iii); impaired stomatal functioning (Preece and Sutter, 1991; Hazarika, 2006). *In vitro* grown plants also have poor photosynthetic efficiency and vascular connection between the shoots and roots. This abnormal morphology, anatomy and physiology of *in vitro* plantlets (Pospisilova et al., 1992, 1997; Buddendorf-Joosten and Woltering, 1994; Desjardins, 1995) make difficult for the plantlets to survive *ex vivo*. In *Gladiolus*, there are very few but varied reports of transplanting *in vitro* grown plants either from direct or indirect regeneration. No optimized protocol has yet been developed for acclimatization process in *Gladiolus*. Ziv (1979) transferred *in vitro* raised propagules on half-strength MS medium supplemented with a reduced sucrose concentration (1.5%), 0.4 mg/L thiamine, 0.5 mg/L NAA and 0.3% activated charcoal, and gown under a higher light intensity than used for maintaining the microporpagated plants. Ziv (1991) also reported that the addition of paclobutrazol to the medium resulted in the formation of cormels with 100% survival following transfer to the greenhouse, whereas 58% was observed without paclobutrazol. Priyakumari and Sheela (2005) reported successful acclimatization of the *Gladiolus* plantlets planted in 2:1 of sand and soil in plastic pots. Earlier Jager et al. (1998) also reported similar results.

In *Lilium speciosum* Thunb. var. gloriosoides Baker, 98% survival rate of rooted plantlets was recorded in 35 cavity growing trays under mist condition for first four weeks (Chang et al., 2000). Hannweg et al. (1996) also found almost same results in *Bowiea volubilis*. They transplanted *in vitro* regenerated plantlets in sterilized soil and washed coarse river sand under three different conditions: (i) Covered tightly for seven days to achieve high relative humidity; (ii) Used loose covering for two to three weeks to acquire medium relative humidity; (iii) Plantlets uncovered and mist sprayed twice daily. Mist sprayed plantlets gave maximum survival rate (90.9%) as compared to other conditions. This phase of transplantation from *in vitro* to *in vivo* usually needs some weeks of acclimatization with gradual lowering in air humidity (Preece and Sutter, 1991; Bolar et al., 1998).

To reduce the losses which occur during the hardening process of *in vitro* grown plants, it is better especially in bulbous plants to induce shoots to form storage organs such as cormels in *Gladiolus* and bulbs of lilies. These underground storage organs are generally resilient and can be planted or stored when desired. However, the survival of *in vitro* plantlets with cormels/bulblets is usually based on the size of the cormels as reported by Naik and Nayak (2005) in *Ornithogalum virens*; Slabbert and Niederwieser (1999) in *Lachenalia.* Smaller bulbs (2 to 3 mm diameter) showed low survival as compared to large one (4 to 10 mm diameter) (Naik and Nayak, 2005). Paek and Murthy (2002) reported that 100% survival of *in vitro* rooted bulblets had diameter of more than 10 mm. Cormels usually undergo dormancy and thus do not sprout upon planting. A cold treatment is followed to break the dormancy of the cormels before plantation of cormels (De Hertogh et al., 1974; Stimart and Ascher, 1982). *Gladiolus* requires cold treatment for a period of four weeks at a temperature range of 2 to 5°C as reported by Hussey (1977). He also reported that dormancy can also be broken when *in vitro* produced cormels are subcultured on a medium containing BA. A period of 4 to 8 weeks at 0 to 5°C was required to break dormancy in bulblets (Bacchetta et al., 2003). Paek and Murthy (2002) employed cold treatment for 5 weeks at 5°C in *Fritillaria thunbergii*.

Role of corm size in acclimatization

Corm size also plays major role in the acclimatization of the bulbous plants as poor survival rate was observed within five to seven days from bulblets having smaller than 4 mm diameter whereas with larger bulblets more survival rate was obtained (Hannweg et al., 1996). Paek and Murthy (2002) also planted *in vitro* regenerated bulblets of *F. thunbergii* of different sizes in equal ratio of peat moss, vermiculite and perlite. They recorded survival rate of 17.6% after five weeks from bulblets having less than 5 mm diameter whereas 86 and 100% was observed from bulblets having 6 to 10 mm and more than 10 mm diameter, respectively. Naik and Nayak (2005) reported that bulblets of small size (2 to 3 mm diameter) had survival rate of 40 to 50%, whereas the larger bulblets (4 to 10 mm diameter) had a 70 to 80% survival rate.

Conclusions

It is concluded from various works done by scientists that corm and comel production can be multiplied successfully from large sized corms as compared to small sized corms. However, commercial producers may be able to use large sized corms for producing both marketable flower spikes as well as corm and cormel production. Regeneration of plantlets and *in vitro* production of cormels was successfully achieved through direct and indirect mode of regeneration. However, production of *in vitro* cormels through direct regeneration procedures seems to be promising for commercial production of corms and the production quality in the future.

Conflict of interests

The authors have not declared any conflict of interests.

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Growth regulators, DNA content and anatomy *in vitro*-cultivated *Curcuma longa* seedlings

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Curcuma longa* L., from the Zingiberaceae family, generally reproduces through its rhizomes, which are also utilized for therapeutic purposes because they are rich in terpenoids. Its conventional propagation has low efficiency due to the small number of seedlings and their contamination by pathogens. Therefore, this study aimed to evaluate the influence of growth regulators on the development of *in vitro*-cultivated *C. longa* as well as to determine their influence on DNA content and foliar anatomy. Shoots were inoculated in MS culture medium with the addition of 30 g/L of sucrose and 6.5 g/L of agar, and a pH adjusted to 5.8. Two assays were built to study the multiplication and rooting phases of growth. The first assay evaluated the influence of eight concentrations of cytokinins and auxins on the multiplication phase. Leaf samples were analyzed for DNA content through flow cytometry, utilizing two reference standards, green pea, and tomato. Characteristics of leaf anatomy were also measured in four time periods. The second assay analyzed the influence of six auxin concentrations on the rooting phase. The first assay showed that the root systems grew more in treatment 3 (4.44 µM benzylaminopurine [BAP], 0.46 µM kinetin [KIN]) and reached greater dry mass in T8 (8.88 µM BAP, 0.92 µM KIN, 2.16 µM naphthalene acetic acid [NAA]). The largest fresh matter of the main shoot was found in T2 (4.44 µM BAP). The estimated DNA content varied depending on the presence of supplemental growth regulators, from 2.38 to 2.77 pg, and was greater in T4 (4.44 µM BAP, 1.08 µM NAA) and T5 (4.44 µM BAP, 0.46 µM KIN, 1.08 µM NAA). Results from the latter two treatments were not significantly different. Estimates of DNA content were precise, as indicated by coefficients of variation that were much lower than 5%. The results also showed quantitative variation of evaluated anatomical traits. In general, there was a thin epidermis layer with rectangular cells, followed by parenchyma with octahedral cells and differentiated xylem and phloem. In leaf parenchyma, the presence of idioblasts containing phenolic compounds was observed in all growth stages. In the rooting phase, the supplementary auxins affected the dry matter of the aerial part and roots. The highest averages were observed in treatments with 2.0 µM NAA.

Key words: Turmeric, micropropagation, flow cytometry, vegetal anatomy.

INTRODUCTION

Curcuma longa L. from the Zingiberaceae family, popularly known as turmeric, is a perennial plant native to Southern and Southeastern Asia (Pinto and Graziano, 2003). It is a medicinal plant whose rhizomes, generally rich in terpenoids, are utilized for therapeutic purposes (Nicoletti et al., 2003). Curcumin is its main compound of

interest (Chainani, 2003). Among the pharmaceutical applications, *C. longa* has anti-inflammatory, antioxidant, and antitumor properties (Kainsa et al., 2012; Green and Mitchell, 2014). It is indicated for the treatment of gastritis, ulcers, and food poisoning, acting mainly on the digestive system. *C. longa* has also been included in the treatment of cancer, hepatitis, inflammations, and painful diseases like arthritis, to name but a few (Mata, 2004). Moreover, this species stands out as a spice and food coloring for pasta and it is used for decoration due to its durability, beauty, and the exuberance of its inflorescences (Costa et al., 2011).

One of the problems *C. longa* producers face is the conventional propagation system. This system is limited by the phytosanitary quality of rhizomes, leading to dissemination of soil pathogens like fungi and nematodes (Bharalee et al., 2005; Faridah et al., 2011). The propagation is long and costly a rhizome can only produce four plants and has a period of dormancy, which is common in Zingiberaceae (Zhang et al., 2011). In addition, this rhizome, necessary for propagation, is also the commercialized part of the plant (Bharalee et al., 2005). Micropropagation could be a possible solution for this problem as *in vitro* methods are frequently utilized to complement traditional methods (Ahmadian et al., 2013). This technique can provide a greater number of pathogen-free seedlings in a shorter amount of time (Yildiz, 2012).

Several protocols have been used, altering the basal media and using different concentrations of growth regulators to meet the needs of each species. Santos (2003) stated that supplementary growth regulators replace the hormones missing from explants isolated from the mother plant. The different types of regulators work as stimuli for the expression of genes that control plant development, resulting in the induction of shoot and root growth and tissue differentiation (Depuydt and Hardtke, 2011). The most utilized vegetal regulators in tissue culture are auxins and cytokines, and among them, 3-indoleacetic acid (IAA), naphthalene acetic acid (NAA), Kinetin (KIN) and benzylaminopurine (BAP) have been the most utilized in assays (Neelakandan and Wang, 2012). Results for other species of Zingiberaceae showed that these regulators were paramount to promote growth and higher numbers of shoots of *Zingiber zerumet* (Faridah et al., 2011) and *Etlingera elatior* (Abdelmageed et al., 2011), and increased rooting rate and root length of *Zingiber officinale* (Abbas et al., 2011), and *Curcuma soloensis* (Zhang et al., 2011).

Tools like flow cytometry (Doležel and Greilhuber, 2010a) and structural and morphological analyses of tissues also help explain the effect of these regulators on seedling development because the regulators influence tissue differentiation (Aloni et al., 2004; Aloni et al., 2006). In addition, studies that anatomically describe Zingiberaceae, including *C. longa*, are scarce. According to Aloni et al. (2006), auxins and cytokinins control the differentiation of xylem and phloem, and other hormones, like gibberellins and ethylene, may also be involved in this process. Assays demonstrated that the addition of BAP along with KIN in the culture medium increased the thickness of parenchyma cells, both spongy and palisade, and consequently the thickness of foliar limbs in *Annona glabra* (Oliveira et al., 2008). The quantitative analysis of leaf tissues of two species in Bromeliaceae showed that 0.5 mg/L of BAP resulted in a greater distance between the xylem and phloem (Galek and Kukulczanka, 1996). Thus, it is expected that, such as in other species of Zingiberaceae, auxins and cytokinins may have an effect on the development and anatomy of *C. longa*.

The ideal propagation protocol promotes better development and health vigorous seedling without the occurrence of abnormalities. However, the excess of growth regulators might be toxic to plant tissue and trigger an abnormal seedling development (Anjanasree et al., 2012). It was observed in *Elaeis guineensis* that the addition of 0.05 NAA + 0.05 BAP + 0.05 GA_3 + 2000 activated carbon (mg/L) (Suranthran et al., 2011).

In grapes, the addition of 10 µmol de GA_3 + 10 µmol IAA in MS medium promoted 56% of abnormal seedlings (Ji et al., 2013). In this way, monitoring through anatomical observations ensure a better understanding about the process, what is usually done visually. In addition, this would help in the comprehension of regulators action on the *C. longa* morphogenesis, once there are few studies about it.

Flow cytometry is used to characterize vegetal material for several purposes, such as DNA content analysis, ploidy verification, and cell cycle acquisition (Ochatt, 2008). Specifically, in tissue culture, this technique has been important to verify genetic stability, identify hybrids, check ploidy, and quantify genome size (Doležel and Greilhuber, 2010b; Pasqual et al., 2012). Growth regulators through *in vitro* culture, may cause somaclonal variations which might be from genetic or epigenetic (Miguem and Marum, 2011; Georgiev et al., 2014); this mechanism regulation influences the genetic expression affecting the phenotype. Furthermore, errors in DNA reading frame, might affect tissue analysis. Recently, researchers have discussed about the occurrence of self-tanning (Bennett et al., 2008) on the tissues *in vitro* analysis. Thus, flow cytometry, may contribute to verify the occurrence of somaclonal variation and is also

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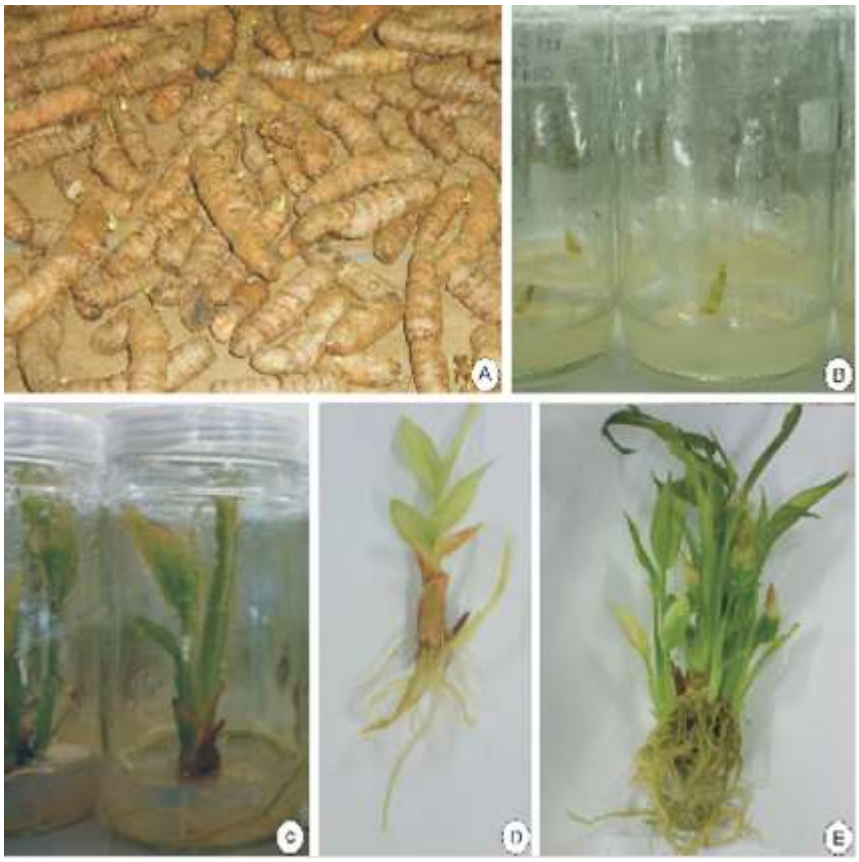

Figure 1.Rhizomes, shootings and developing seedlings of *C. longa* in function of the concentration of cytokinins and auxins. (A= Rhizomes, B= Inoculated shootings, C= developing seedlings, D=seedling submitted to Treatment 2 and E= seedling submitted to Treatment 8).

important to the monitoring of self-tanning in *C. longa* in studies with growth regulators.

Thus, the aim of this study was to evaluate the influence of growth regulators on the development of *in vitro C. longa*, as well as to verify the DNA contents and foliar anatomy of this species.

MATERIALS AND METHODS

C. longa rhizomes were obtained in the city of Mara Rosa (Figure 1A), GO, Brazil, and transported in bags to the Laboratory of Molecular Biology and Vegetal Tissue Culture of Paranaense University (UNIPAR), campus of Umuarama, PR, Brazil.

Shoot asepsis

The rhizomes were kept in the laboratory for a month at room temperature prior to selection for culture. Rhizomes that were cracked or had symptoms of infection by pathogens were discarded. The remaining rhizomes were washed in running water to remove soil fragments. After emergence, the shoots were removed from the rhizomes and standardized to 2.0 ± 0.2 cm in length and 0.5 ± 0.2 cm in diameter. In an aseptic chamber, the shoots were immersed in a solution of 2% (v/v^{-1}) sodium hypochlorite for 20 min under manual agitation and then submitted to three successive washings in distilled water.

Phase 1: Multiplication phase

Axillary shoots from the asepsis phase were inoculated in 350 ml clear glass flasks (Figure 1B) containing MS culture medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g/L of sucrose and 6.0 g/L of agar, and adjusted to a pH of 5.8. Three growth regulators, BAP, NAA, and KIN, were added to the culture medium at different concentrations (Table 1). Inoculation was done in an aseptic chamber after autoclaving of the flasks at 121°C for 20 min. The shoots were individually placed in flasks with 50 ml of culture medium. The flasks were then closed with transparent plastic lids and sealed with PVC plastic. The material was kept in a growth chamber for 101 days at a temperature of 25 ± 2°C, in the presence of light for 24 h per day.

After 101 days, the following characteristics were evaluated: leaf number (LN), shoot number (SN), aerial part length (APL), base diameter (BAD), root length (RL), fresh matter of main shoot (FMMS), root fresh matter (RFM), total dry matter of aerial part (DMAP), and root dry matter (RDM). Data for length were measured by a digital pachymeter and dry matter measurements were obtained after drying in an air circulation oven at 65°C until measurements were constant.

The experiment had a complete randomized design with eight

Table 1. Concentration of different growth regulators, BAP, KIN and NAA added to the culture medium (MS).

Treatment	BAP (µM)	KIN (µM)	NAA (µM)
T1	0.0	0.0	0.0
T2	4.44	0.0	0.0
T3	4.44	0.46	0.0
T4	4.44	0.0	1.08
T5	4.44	0.46	1.08
T6	8.88	0.0	0.0
T7	8.88	0.92	0.0
T8	8.88	0.92	2.16

treatments, one shoot in each flask, three shoots per plot, and four replicates. Leaf number, base diameter, and aerial part length were submitted to an analysis of variance by a Kruskal Wallis test (p≤0.05), whereas the other traits were submitted to an analysis of variance (ANOVA, p≤0.05). The averages were compared using Tukey's test (p≤0.05).

DNA content estimate by flow cytometry

At 101 days after assay implementation, a leaf portion of approximately 1 cm from each treatment was removed and ground in a Petri dish with 1 ml cold Marie buffer in order to release nuclei (Marie and Brown, 1993). The buffer solution consisted of 50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM Na_2 EDTA, 50 mM sodium citrate, 0.5% Tween 20, 50 mM HEPES (pH 7.2), and 1% (m/v) polyvinylpyrrolidone-10 (PVP-10). The nuclei suspension was aspirated through two layers of cotton gauze using a plastic pipette and filtered through a 50-µm mesh. The nuclei were then stained by adding 25 µl of 1 mg/ml propidium iodide to each sample.

To compare DNA content in picograms, two other species, *Pisum sativum* with 9.09 pg (Pasqual et al., 2012) and *Solanum lycopersicum* with 1.86 pg were used as external reference standards, using the same procedure for nucleus suspension. For each sample, 10,000 nuclei were evaluated through a logarithmic scale. The analysis was carried out in a FACS Calibur cytometer (BD, Biosciences, San Jose, CA, USA) and the histograms were obtained by Cell Quest software and statistically analyzed by WinMDI 2.8 software (Scripps 43 Research Institute, 2011). Nuclear DNA content (pg) was estimated as the ratio between the fluorescence intensities of G1 nuclei from the reference standard and G1 nuclei from the sample, multiplying this ratio by the DNA amount of the reference standard. Estimated DNA contents and coefficients of variation (CV%) were submitted to an analysis of variance and the averages were compared using Tukey's test (p≤0.05) in Sisvar statistical program (Ferreira, 2011). All analyses were done in quadruplicates.

Anatomical evaluation

Samples of the vegetal material from each of the eight treatments in the multiplication phase were collected at different periods. Samples for period 1 were collected immediately after *in vitro* inoculation; period 2 at 35 days after inoculation; period 3 at 56 days; and period 4 at 101 days.

Each sample was fixed in FAA 50 solution (formaldehyde, 50% ethanol, acetic acid, 1:1:18, v/v) for 24 h and stored in 70% ethanol (Johansen, 1940). For permanent slide preparation, the material was dehydrated in a butylic series (Johansen, 1940) and embedded in Paraplast (Kraus and Arduin, 1997) in an oven at 60°C. Transversal cuts (7 µm) were done in a rotary microtome at the Pathology Histotechnical Laboratory of Unipar – Campus 2. The histological cuts were placed in a hot water bath at 45°C and immediately removed with glass slides. The slides with fixed vegetal material were placed in a water bath in butyl acetate to remove the excess Paraplast. For complete removal of Paraplast the samples were dehydrated in an ethylic series. Next, they were stained with safrablau, a mixture of astra blue and safranin (9:1, v/v) modified to 0.5% (Bukatsch, 1972). Acrilex colorless glass varnish was used for adhesion of coverslips (Paiva, 2006).

The prepared slides were utilized to measure epidermis, parenchyma, xylem, phloem of the aerial part of the plant. These measurements were made from images of the longitudinal sections captured by a digital camera coupled to an optical light microscope, Olympus BX-60, using Motic Images Plus 2.0 software. To calculate averages, 10 cells from each slide were used in three replicates divided into the aerial part for each of the following variables: epidermis, parenchyma, xylem, and phloem. The averages were compared by Scott-Knot's test (p≤0.05).

Table 2. Concentration of different growth regulators, NAA and IAA, added to the culture medium (MS).

Treatment	NAA (µM)	IAA (µM)
1	0.0	0.0
2	1.0	0.0
3	2.0	0.0
4	0.0	34.0
5	0.0	44.0
6	1.0	34.0

Phase 2: Rooting phase

Aseptic shoots were inoculated in MS culture medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g/L of sucrose and 6.0 g/L of agar, and adjusted to a pH of 5.8. Two growth regulators, NAA and indoleacetic acid (IAA), were added to the culture medium at different concentrations (Table 2). The culture media were autoclaved at 121°C for 20 min.

In an aseptic chamber, the shoots were individually placed in flasks containing 50 ml of culture medium, closed with clear plastic lids, and sealed with PVC plastic. The material was kept in a growth chamber for 60 days at a temperature of 25 ± 2°C and submitted to 24 h of light per day.

After 60 days, the following characteristics were evaluated: leaf number (LN), base diameter in mm (BAD), root length in mm (RL), fresh matter of main shoot (FMMS), root fresh matter (RFM), shoot number (SN), dry matter of aerial part (DMAP), and root dry matter (RDM). Data for length were measured by a digital pachymeter. Dry matter measurements were obtained after drying in an air circulation oven at 65°C until measurements were constant. The experiment had completely randomized design (CRD) with six treatments, three shoots per plot, and four replicates. Data were submitted to an ANOVA (p ≤0.05), and averages were compared by Tukey's test (p≤0.05).

RESULTS

Phase 1: Multiplication phase

There were significant differences for several of the

Table 3. Growth measurements obtained from *in vitro Curcuma longa* seedlings cultivated with differing concentrations of auxins and cytokinins in the multiplication phase.

Treatment	LN	SN	BAD (mm)	APL (mm)	RL (mm)	FMMS (g)	RFM (g)	DMAP (g)	RDM (g)
T1	7.75^{a}	1.5^{a}	8.12^{a}	67.28^{ab}	54.49^{ab}	1.07^{b}	1.23^{b}	0.10^{a}	0.08^{b}
T2	7.25^{a}	0.75^{a}	5.13^{a}	36.11^{b}	38.94^{ab}	13.31^{a}	2.32^{ab}	0.14^{a}	0.08^{b}
T3	5.75^{a}	2.5^{a}	6.39^{a}	69.72^{ab}	65.19^{a}	1.08^{b}	2.28^{ab}	0.11^{a}	0.12^{b}
T4	6.25^{a}	1^{a}	7.89^{a}	88.92^{a}	54.49^{ab}	1.13^{b}	3.07^{ab}	0.10^{a}	0.17^{ab}
T5	8.25^{a}	2.25^{a}	9.62^{a}	55.99^{ab}	27.58 ^{b}	1.29^{b}	5.42^{ab}	0.12^{a}	0.31^{ab}
T6	3.25^{a}	0.75^{a}	5.82^{a}	38.36^{ab}	30.09 ^{b}	0.44^{b}	2.26^{ab}	0.08^{a}	0.16^{ab}
T7	5.5^{a}	0.75^{a}	6.92^{a}	68.36^{ab}	65.79 ^{a}	1.22^{b}	4.09^{ab}	0.12^{a}	0.14^{ab}
T8	10.75^{a}	2.5^{a}	4.64^{a}	69.72^{ab}	33.17^{ab}	2.29^{b}	7.77^{a}	0.26^{a}	0.36^{a}

*Analysis by Kruskal Wallis' test, (LN) test value=8.472, p (0.05)=14.070; (APL) test value=14.205, p (0.05)=14.070; (BAD)=test value=7.983, p (0.05)=14.070. *Other characteristics, averages followed by the same letter do not differ statistically by Tukey's test at p<0.05. LN: Leaf number; SN: shoot number; APL: aerial part length; BAD: base diameter; RL: root length; FMMS: fresh matter of main shoot; RFM: root fresh matter; DMAP: total dry matter of aerial part; RDM: root dry matter. T1: Control, T2: 4.44 µM BAP, T3: 4.44 µM BAP +0.46 µM KIN, T4: 4.44 µM BAP +1.08 µM NAA, T5: 4.44 µM BAP + 0.46 µM KIN + 1.08 µM NAA, T6: 8.88 µM BAP, T7: 8.88 µM BAP + 0.92 µM KIN, and T8: 8.88 µM BAP + 0.92 µM KIN +2.16 µM NAA.

evaluated growth characteristics (p≤0.05), depending on the types and concentrations of growth regulators. Leaf and shoot number, base diameter, and dry matter of aerial part did not show significant differences (Table 3). However, root and aerial part lengths, fresh matter of main shoot, and root fresh and dry matter were influenced by growth regulators (Table 3).

The treatments that caused greater root growth were treatment 3 (4.44 µM of BAP and 0.46 µM of KIN) and T7 (8.8 µM of BAP and 0.92 µM of KIN), whereas T5 and T6 were the least efficient for root growth. The highest average aerial part length was seen in T4 (4.44 µM/L of BAP added with 1.08 µM/L of NAA). Treatment 2 (4.44 µM of BAP) was the most efficient for increasing the fresh matter of the main shoot. The final averages were similar for the other treatments. Treatment 8 was the most effective for improving root mass; T4, T5, and T6 did not differ among themselves for root fresh matter (RFM) and root dry matter (RDM), whereas T1 was less efficient for RFM. Treatment 1, T2, and T3 were inefficient for improving root mass (Table 3).

Regarding RFM and RDM, high concentrations of cytokinins combined with auxins, such as in T8, caused a decrease of root growth, but favored mass gain with the emergence of several lateral and secondary roots (Figure 1E). Skala and Wysokinska (2004) and Garlet et al. (2011) also observed root length reduction in *Salvia nemorosa* L. and *Mentha gracilis* S. plants when higher concentrations of BAP were used during *in vitro* proliferation of these species. The combination of cytokinins and auxin (NAA) was important for the root system, making synergism between these regulators evident. Treatments with low concentrations of NAA had fewer lateral roots and the control treatment showed the lowest root mass, probably due to the absence of growth regulators.

Figure 2 shows the tendency of the length development of aerial part and root of *C. Longa* seedlings submitted to combinations and concentrations of cytokinins and auxins. For root length, an increment of approximately 40 mm was observed in the first 35 days; this behavior was similar to the other treatments. After that, there was a growth distinction for the aerial part which was influenced by the regulator and its concentration. After 56 days, the treatments with 4.44 µM of BAP added with 0.46 µM of KIN, and 8.88 µM/L of BAP added with 0.92 µM/L of KIN provided length of approximately 52 mm, and at the end of the assay, there was a more evident growth, but a smaller increase for the aerial part in the treatment with 8.88 µM/L of BAP (Figures 1D and 2). Regarding root length, no similar root growth standard was observed for the aerial part in the first 35 days; the control stood out from the others, reaching 65 mm, and the roots in T3 and T7 developed more than in other treatments after 56 days (Figure 2). At 101 days, there was an increase of 32.01 mm in root length when comparing T3 to T8, but root length was 35.1 mm in T3 (Figure 2).

DNA content varied according to treatment for *in vitro*-cultivated *C. longa*. All treatments presented CVs lower than 5% and, consequently, highly reliable DNA indexes (Table 4). Sample quality can influence CV; a lower value indicates greater reliability in the estimates. The highest DNA content was observed in T4 and T5, and the smallest in T3. The results were the same regardless of whether *P. sativum* or *S. lycopersicum* were used as the reference (Table 4).

Figures 3 and 4 provide histograms of flow cytometry using each reference standard. In the histogram of Figure 3, the first peak indicates peak G1 of the interphase of leaves from the experimental treatments whereas the second one indicates peak G1 of the reference standard (*P. sativum*). In Figure 4, the first peak indicates peak G1

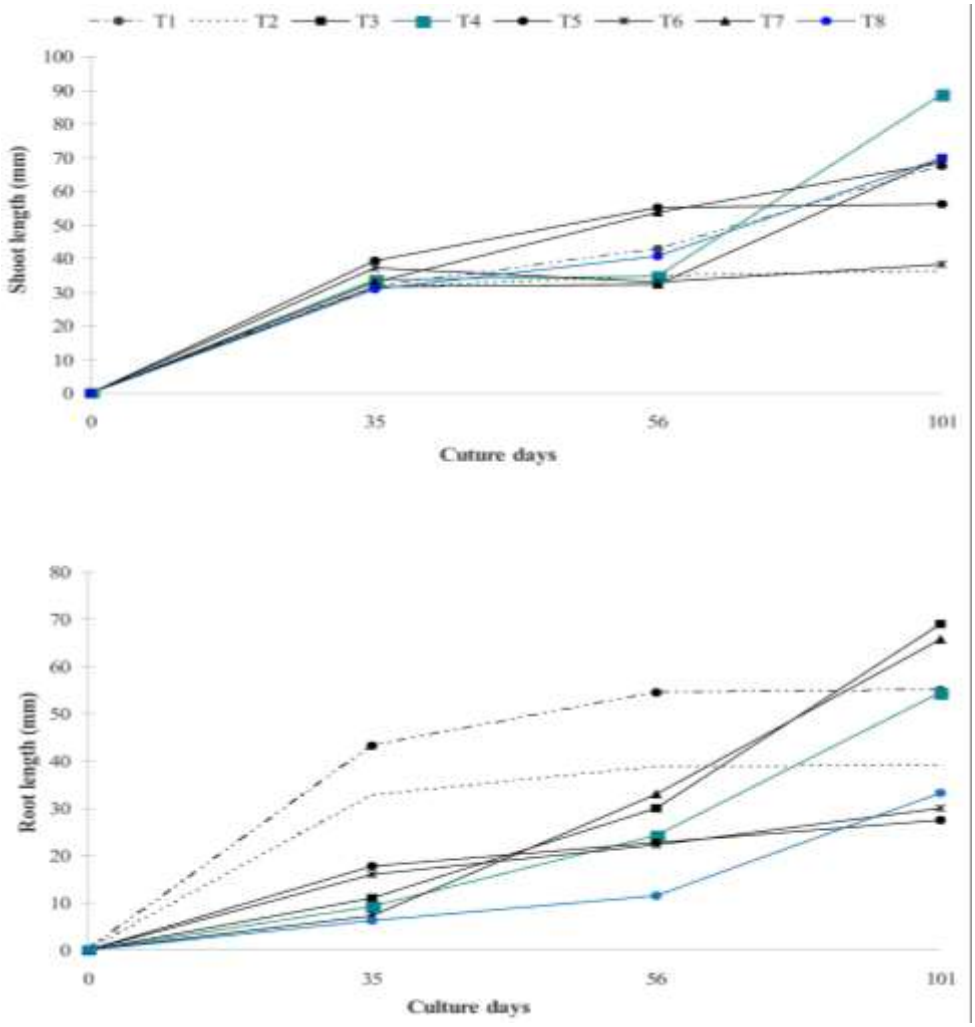

Figure 2. Aerial part length (mm) and root length (mm) evaluated in four distinct periods and at different concentrations of auxins and cytokinins during multiplication phase. **T1**: Control, **T2**: 4.44 (µM/L) of BAP, **T3**: 4.44 **(**µM/L) of BAP+0.46 (µM/L) of KIN, **T4**: 4.44 (µM/L) of BAP+1.08 (µM/L) of NAA, **T5**: 4.44 (µM/L) of BAP+0.46 (µM/L) of KIN+1.08 (µM/L) of NAA, **T6**: 8.88 (µM/L) of BAP, **T7**: 8.88 (µM/L) of BAP+0.92 (µM/L) of KIN, and**T8**: 8.88 (µM/L) of BAP+0.92 (µM/L) of KIN+2.16 (µM/L) of NAA.

Table 4. DNA content and Coefficient of Variation (CV) of *in vitro*- cultivated *Curcuma longa*.

Treatment	Green pea (*Pisum sativum*. L)		Tomato (*Solanum lycopersicum*)	
	DNA (pg)	CV (%)	DNA (pg)	CV (%)
1	2.4450^{ab}	0.49^{a}	2.7025^{ab}	0.49^{a}
2	2.4650^{ab}	0.49^{a}	2.7300^{ab}	0.49^{a}
3	2.3850^{b}	0.49^{a}	2.6275^{b}	0.49^{a}
4	2.5275^{a}	0.49^{a}	2.7775^{a}	0.50^{a}
5	2.5300^{a}	0.48^{a}	2.7575^{a}	0.50^{a}
6	2.4500^{ab}	0.48^{a}	2.6825^{ab}	0.50^{a}
7	2.4950^{ab}	0.48^{a}	2.7500^{ab}	0.49^{a}
8	2.4500^{ab}	0.46^{a}	2.7125^{ab}	0.48^{a}

*Averages followed by the same letter do not statistically differ among themselves by Tukey's test at p(<0.05). T1: Control, T2: 4.44 µM BAP, T3: 4.44 µM BAP+0.46 µM KIN, T4: 4.44 µM BAP+1.08 µM NAA, T5: 4.44 µM BAP+0.46 µM KIN+1.08 µM NAA, T6: 8.88 µM BAP, T7: 8.88 µM BAP+0.92 µM KIN, and T8: 8.88 µM BAP+0.92 µM KIN+2.16 µM NAA.

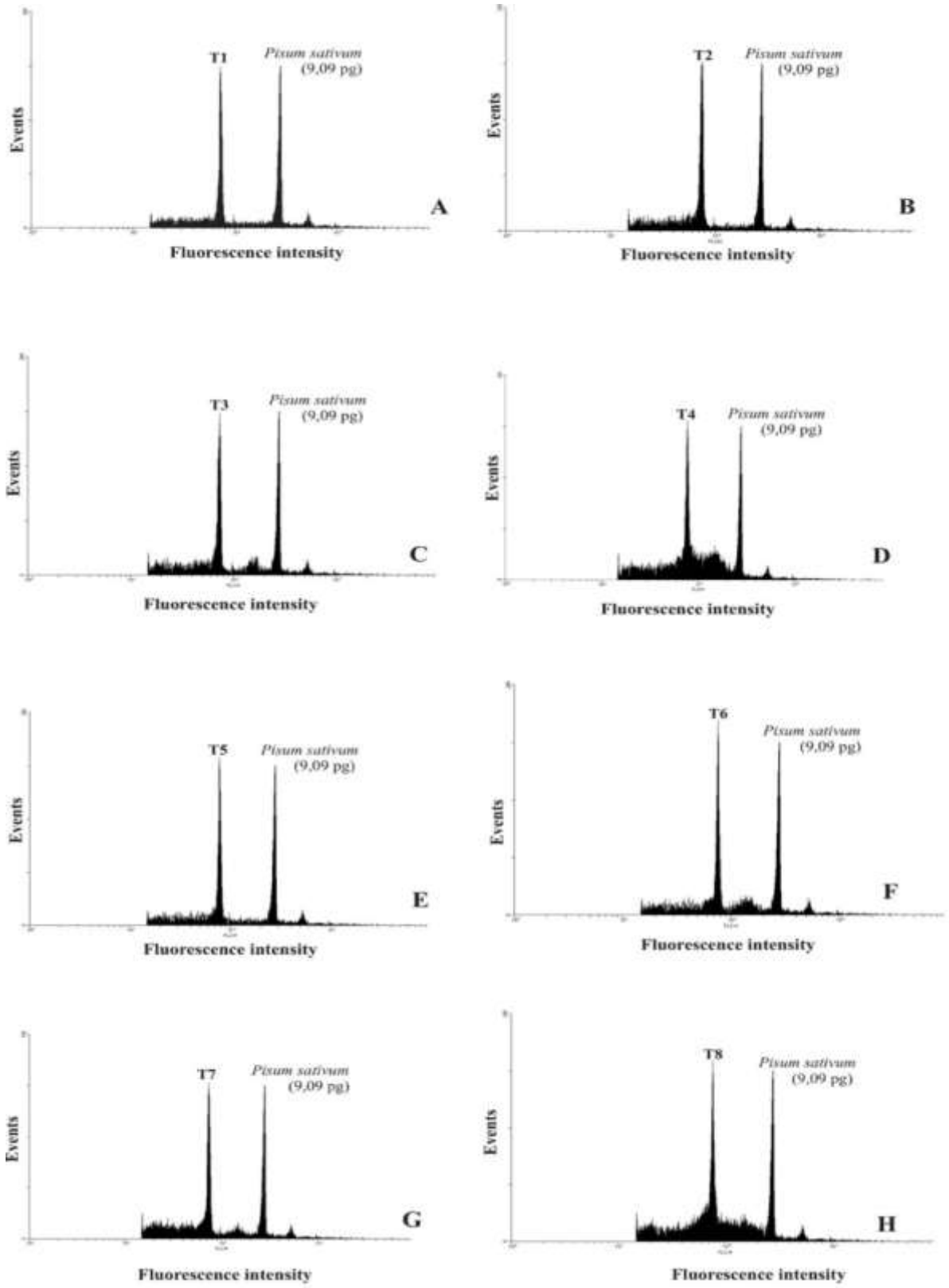

Figure 3. Histograms of flow cytometry of *Curcuma longa* leaves grown with different combinations and concentrations of auxins and cytokinins in the multiplication phase (green pea reference standard). A=T1: Control, B=T2: 4.44 µM BAP, C=T3: 4.44 µM BAP+0.46 µM KIN, D=T4: 4.44 µM BAP+1.08 µM NAA, E=T5: 4.44 µM BAP+0.46 µM KIN+1.08 µM NAA, F=T6: 8.88 µM BAP, G=T7: 8.88 µM BAP+0.92 µM KIN, and H=T8: 8.88 µM BAP+0.92 µM KIN+2.16 µM NAA.

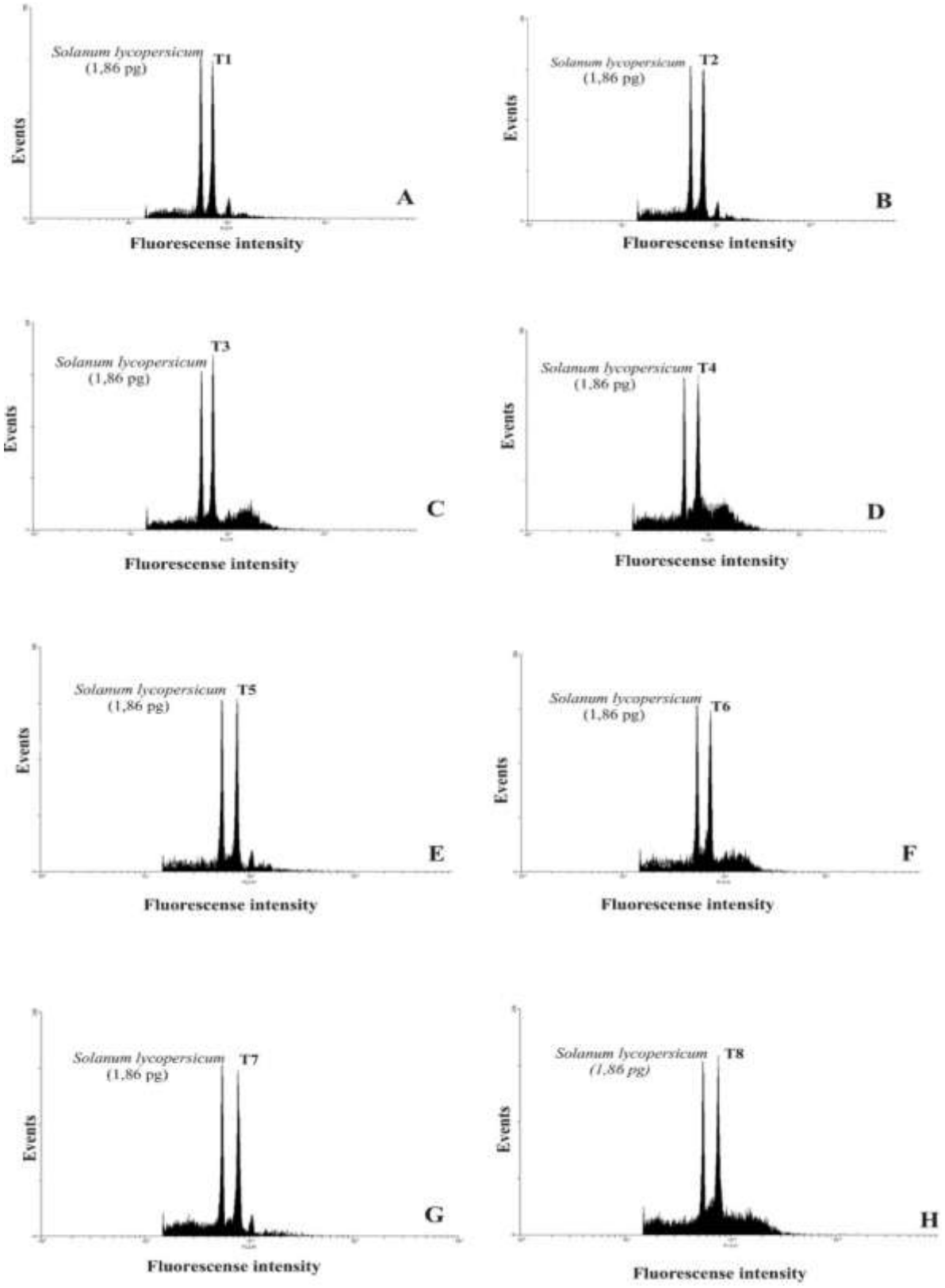

Figure 4. Histograms of flow cytometry of *Curcuma longa* leaves grown with different combinations and concentrations of auxins and cytokinins in the multiplication phase (tomato reference standard). A=T1: Control, B=T2: 4.44 µM BAP, C=T3: 4.44 µM BAP +0.46 µM KIN, D=T4: 4.44 µM BAP+1.08 µM NAA, =T5: 4.44 µM BAP+0.46 µM KIN+1.08 µM NAA, F=T6: 8.88 µM BAP, G=T7: 8.88 µM BAP+0.92 µM KIN, and H=T8: 8.88 µM BAP+0.92 µM KIN+2.16 µM NAA.

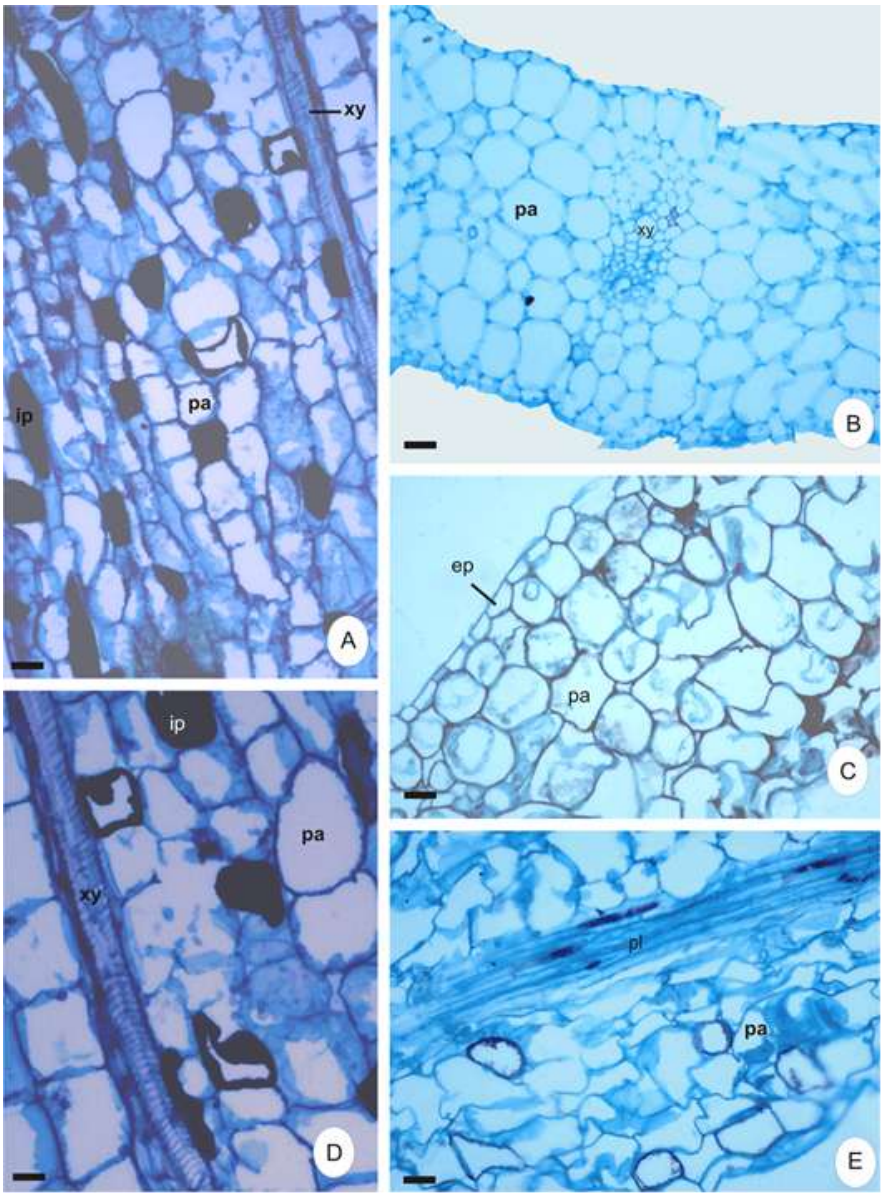

Figure 5. Longitudinal sections of seedling tissues from initiation of the multiplication phase of *in vitro*-cultivated *Curcuma longa*. (A - D) Aerial parts; (E,F) Root. Ep: Epidermis, Pl: phloem, IP: idioblast containing phenolic compounds, Pa: parenchyma, Xy: xylem.

of the reference standard (*S. lycopersicum*), while the second peak indicates the G1 phase of the interphase of leaves from the experimental treatments. In general, the graphs were of excellent quality with thin peaks and little dragging, consistent with the low CV for all treatments and standards.

Regarding to seedling development and anatomical cuts, tissue abnormalities was not verified in treatments with development regulators analyzed. However, in quantitative terms, some differences was observed mainly in parenchyma and in the epidermis. In our study, the epidermis of *C. longa* presented juxtaposed rectangular cells, with thin cell walls with little or no wax (Figure 5A). These results corroborate the ones by Thong et al. (2009) for *C. longa* seedlings kept *in vitro* in 2-4D medium. Epidermis thickness varied according to the treatment and time period (Table 5). At 35 days after cultivation, T4, T5, T7, and T8 had the thickest epidermis cells and were not statistically different from each other. At 56 and 101 days, T2, T4, T6, and T8 had the highest average thickness (Table 5).

The parenchyma of *C. longa* consisted of voluminous cells along both sides of the leaves; the tissue had thin cell walls and the cells had an isodiametric shape with

Table 5. Measurements in µm of longitudinal sections of epidermis, parenchyma, xylem, and phloem of *Curcuma longa* leaves submitted to eight combinations of auxins and cytokinins in the multiplication phase.

Treatments	Epidermis	Parenchyma	Xylem	Phloem
	Aerial part period/2			
1	635,5[b]	915,1[c]	508,9[a]	1.485,0[a]
2	553,3[b]	1.079,7[c]	450,6[a]	1.084,8[b]
3	465,5[b]	1.202,2[c]	348,1[b]	1.345,1[a]
4	784,7[a]	1.523,4[b]	397,5[b]	1.496,0[a]
5	705,6[a]	1.483,9[b]	431,4[a]	1.346,4[a]
6	598,2[b]	1.855,8[a]	466,0[a]	1.626,3[a]
7	711,1[a]	1.962,1[a]	495,6[a]	1.503,8[a]
8	747,6[a]	1.164,5[c]	368,8[b]	1.504,0[a]
Period 1	757,3	1.734,7	519,1	1.783,1
	Aerial part period/3			
1	471,6[b]	1.155,1[c]	418,7[a]	1.377,1[b]
2	733,2[a]	1.961,7[a]	471,8[a]	1.383,5[b]
3	534,3[b]	920,0[c]	409,9[a]	1.161,6[b]
4	656,9[a]	1.441,3[b]	464,3[a]	1.600,5[a]
5	501,6[b]	1.462,2[b]	478,1[a]	1.863,8[a]
6	697,2[a]	1.644,3[b]	390,8[a]	1.289,3[b]
7	443,7[b]	1.319,1[c]	410,5[a]	1.651,8[a]
8	700,9[a]	1.475,5[b]	492,7[a]	1.499,5[b]
Period 1	757,3	1.734,7	519,1	1.783,1
	Aerial part period/4			
1	471,6[b]	1.155,1[c]	385,5[a]	1.377,1[b]
2	733,2[a]	1.961,7[a]	526,7[a]	1.383,5[b]
3	534,3[b]	920,0[c]	466,8[a]	1.161,6[b]
4	656,9[a]	1.441,3[b]	497,4[a]	1.600,5[a]
5	501,6[b]	1.462,2[b]	443,6[a]	1.863,8[a]
6	697,2[a]	1.644,3[b]	537,1[a]	1.289,3[b]
7	443,7[b]	1.319,1[c]	375,7[a]	1.651,8[a]
8	700,9[a]	1.475,5[b]	545,8[a]	1.499,5[b]
Period 1	757,3	1.734,7	519,1	1.783,1

*Averages followed by the same letter are not significantly different according to Skott-Knott's test (p≤0.05). Period 1: assay implementation, period 2: 35 days after cultivation, period 3: 56 days, and period 4: 101 days.

small intercellular spaces observed in the longitudinal sections (Figure 5B to C). After 35 days in the culture medium, the cells became bigger with more intercellular spaces, and the cell walls got thicker. Idioblasts with phenolic compounds were observed in the leaf parenchyma of the seedlings utilized at the beginning of the assay (Figure 5A and D). The parenchyma, as well as the epidermis, presented significant differences quantitatively among the studied treatments and time periods. The greatest averages at 35 days obtained in this tissue were observed in T6 and T8, whereas T2 had the greatest average at 56 and 101 days (Table 5).

The only significant differences in xylem thickness in the present study were found at 35 days, with differences in the averages for T1, T2, T5, T6, and T7 (Table 5). The phloem had already been differentiated at the assay implementation; at that time, T2 had a significantly lower average phloem thickness compared to the rest of the treatments. At 56 and 101 days, T2 remained the lowest, whereas T3, T4, and T7 had significantly higher phloem thickness compared to the rest of the treatments (Table 5).

Table 6. Growth measurements obtained from *in vitro Curcuma longa* seedlings cultivated with differing concentrations and combinations of auxins and cytokinins in the rooting phase.

Treatments	SN	LN	APL (mm)	RL (mm)	BAD (mm)	FMMS (g)	FMAP (g)	RFM (g)	DMAP (g)	RDM (g)
1	1.^{20b}	8.33^{a}	68.24^{a}	119.24^{a}	8.99^{ab}	2.18^{a}	2.67^{a}	0.1596^{a}	0.1160^{c}	2.3060^{bc}
2	1.20^{b}	8.56^{a}	63.24^{ab}	55.97^{b}	8.17^{b}	2.24^{a}	2.80^{a}	0.1648^{a}	0.1340^{bc}	2.1580^{c}
3	1.80^{ab}	8.95^{a}	58.55^{ab}	72.39^{b}	9.82^{ab}	2.33^{a}	2.94^{a}	0.1715^{a}	0.2300^{a}	4.4120^{a}
4	2.20^{a}	8.93^{a}	52.86^{b}	74.08^{b}	9.16^{ab}	2.33^{a}	3.04^{a}	0.1786^{a}	0.2080^{a}	2.5780^{bc}
5	1.80^{ab}	8.90^{a}	58.03^{ab}	77.80^{b}	10.95^{ab}	2.54^{a}	3.13^{a}	0.1784^{a}	0.2080^{a}	3.8160^{ab}
6	1.60^{ab}	8.80^{a}	71.74^{a}	81.56^{b}	11.72^{a}	2.63^{a}	3.25^{a}	0.1780^{a}	0.1960^{ab}	3.6520^{abc}

*Averages followed by the same letter do not differ statistically by Tukey's test at p<0.05. LN: Leaf number, SN: shoot number, APL: aerial part length, RL: root length, BAD: base diameter, FMMS: fresh matter of main shoot, FMAP: fresh matter of aerial part, RFM: root fresh matter, DMAP: dry matter of aerial part, RDM: root dry matter. T1: Control (absence of regulator), T2: 1.0 (µM L) NAA, T3: 2.0 (µM L) NAA,T4: 34.0 (µM L) IAA, T5: 44.0 (µM L) IAAandT6: 1.0 (µM L) NAA added with 34.0 (µM L) IAA.

Phase 2: Rooting phase

The results showed significant differences among the six treatments of this phase ($p \leq 0.05$) for the following characteristics: number of shoots, length of aerial part and root, base diameter, and dry matter of aerial part and root. The rest of the characteristics did not vary in response to changes in supplemental regulators (Table 6). The largest final shoot diameter was seen in T6 (1.0 µM NAA+ 34.0 µM IAA). However, the highest shoot number (SN) was found in T4 (34.0 µM/L IAA), followed by T5 (44.0 µM IAA), and T6 (1.0 µM NAA + 34.0 µM IAA). The control and T2 resulted in the lowest averages (Table 6). The longest aerial part length (APL) values were seen in the control (T1) and T6 (1.0 µM NAA+ 34.0 µM IAA; Table 6) and were similar between those two treatments.

It is important to point out that the root system of *C. longa* formed several lateral and secondary roots and large amounts of root hair; therefore, the influence of regulators on growth was also measured using dry mass. The largest values for the dry matter of aerial part (DMAP) were found in three treatments, T3 (2.0 µM NAA), T4 (34.0 µM IAA), and T5 (44.0 µM IAA; Table 6). The DMAP was twice as small in the control treatment compared to T3, suggesting the importance of supplementary growth regulators (Table 6). Only isolated auxin, 2.0 µM of IAA, was efficient enough to increase RDM, since the lowest average was observed in T2 (Table 6).

DISCUSSION

Phase 1: Multiplication phase

C. longa shoots presented a well-developed root system with many lateral roots and an aerial part with a great number of leaves (Figure 1E). Depuydt and Hardtke (2011) reported that different plant organs do not necessarily respond similarly to the action of growth regulators, and these responses can occur distinctly depending on the regulator. The cytokinin BA influences mitosis, whereas auxins affect DNA replication; combined, the two types of growth regulators regulate cell division (Nishiyama et al., 2011; Simon and Petrášek, 2011). This is a complex mechanism since these regulators act as signals for gene expression during development (Depuydt and Hardtke, 2011).

The most utilized growth regulators in assays within Zingiberaceae have been BAP, NAA, and KIN; however, other combinations could be tested in future assays. With concentrations close to the ones used in our study, Prakash et al. (2004) obtained high shoot growth rates in *Curcuma amada*, because a medium supplemented with (4.44 µM BAP + 1.08 µM NAA) and another to (8.88 µM BAP + 2.70 µM NAA) resulted in 80 and 72% regeneration, respectively when compared with a medium without growth regulators. In *Kaempferia galangal*, 8.87 µM of BAP added with 2.46 µM of IBA resulted in an average of 8.3 shoots (Chithra et al., 2005). However, Yunus et al. (2012), utilizing 13.32 µM BAP in *E. elatior*, observed two 3.15-cm shoots and approximately 3 leaves after 12 weeks of cultivation. A significant difference in leaf number among the treatments was not found in our study, and the average across all treatments was 6.5 leaves per shoot.

The results for these species indicated better outcomes with the utilization of only BAP, whereas the combination with an auxin is necessary in other species (Nayak, 2000; Bharalee et al., 2005; Kambaska and Santilata, 2009). In our assay, the addition of BAP led to greater gain in mass for the largest shoot (Figure 1D), improved root length when combined with KIN, and increased root mass when combined with NAA. The superiority of BAP for inducing responses, especially in shoots, can either be attributed to the capacity of the plant tissues to metabolize it more easily than other growth regulators, or to the capacity of this substance to induce the production of endogenous hormones like zeatin (Varshney et al., 2013). However, when comparing the control treatment to the others, it was observed that the average values for leaf and shoot

numbers were not significantly different. In the case of these two traits, the exogenous dose may not have been enough to induce endogenous hormones and, consequently, there was no response. Yunus et al. (2012) similarly verified that shoots had high survival rates and equal average shoot numbers in treatments with and without supplemental growth regulators in *E. elatior*. The same was observed for *C. longa* (Jala, 2012) and *Matthiola incana* (Kaviani, 2014).

Recently, flow cytometry has been used in the assays which evolves tissue culture, mainly when evolves growth development, where there is the possibility of occurrence of somaclonal variation in tissues.

Recently, flow cytometry has been used in assays involving tissue culture (Miguem and Marum, 2011) and self-tanning (Bennett et al., 2008). Moreover, studies utilizing flow cytometry for members of the Zingiberaceae family are scarce. For *C. longa*, DNA content ranged from 2.38 to 2.77 pg, regardless of the utilized standard. Across *Curcuma*, some variation in DNA content has been found depending on the studied species. Islam (2004) reported that *C. amada* varied from 3.2 to 5.3 pg, whereas *Curcuma angustifolia* and *Curcuma attenuata* reached 3.28 and 3.18 pg, respectively. The values obtained for these diploid species are higher than for *C. longa* (2n) and genome size is greater yet for polyploid species like *Curcuma malabarica*, varying from 4.30 to 8.84 pg (Nayak et al., 2006).

Estimates of DNA content in the present study varied slightly depending on the treatment. Possible explanations for this include intrinsic factors of the vegetal matter, such as hormonal interference and a possible induction of somaclonal variation (Miguel and Marum, 2011) and external factors that involve sample preparation prior to the cytometer readings (Camolesi et al., 2007); somaclonal variation may induce morphologica, physiological and biochemical variation (Miguel and Marum, 2011; Georgiev et al., 2014) which may cause positive or negative characteristics. In morphology, the main alteration are the occurrence of variagations which is common in ornamental plants and in pineapple (Rodrigues et al., 2007) or with over growth as in *Musa acuminata* cv *prata-anã* (Albany et al., 2005). In this study, DNA variations might be associated to these phenomena. However, future assays will be necessary to prove it and when detected, to identify which type of alterations was induced.

Some authors suggest that chemicals in the tissue can interfere with the analysis (Bennett et al., 2008). A phenomenon known as self-tanning weaves phenolic compounds into the DNA, resulting in reading errors in the equipment (Doležel and Greilhuber, 2007). In our study, the chemical composition of *C. longa* was not analyzed; however, numerous phenolic idioblasts were observed in foliar tissues (Figure 5A to D) and they may have caused the small variation in measurements.

DNA content in the absence of supplemental growth regulators was larger or smaller from that seen for the other treatments, regardless of the utilized reference standard. The presence of idioblasts was observed even in the control treatment (Figure 5A); they occur naturally in this species. Although T2, T3, T7, and T8 resulted in the greatest shoot and root length and root mass, T4 and T5 showed the highest DNA content. The same result was reported for *Butia capita*, in which idioblasts containing phenolic compounds were observed in all development phases of *in vitro* plants. Analysis by flow cytometry showed changes in DNA content at various stages of seedling development except at the embryo stage; the authors attributed the presence of idioblasts containing phenolic compounds as responsible for altering the DNA content (Magalhães et al., 2015).

Tannins have been noted as the main chemical compounds that interfere with cytometer readings (Loureiro et al., 2006; Doležel and Greilhuber, 2007; Bennett et al., 2008). However, these same authors reported that DNA content can be estimated by cytometry without problems if one takes precautions. To mitigate the effect of these compounds, extraction buffers can be used to promote a greater removal of phenolic compounds (Loureiro et al., 2006), including Galbraith's buffer and LB01 (Galbraith et al., 1983; Doležel et al., 1989), Otto buffer, and other adaptations (Otto, 1990; Doležel and Göhde, 1995). Other vegetal materials like dry roots and seeds (Jedrzejczyk and Sliwinska, 2010) can also be used for analyses, but colored tissues such as flowers and fruits should be avoided (Bennett et al., 2008).

Due the importance of the influence of growth regulators in plant development in our assay, it was visually observed that the treatments did not cause abnormal seedling formation, what was confirmed in anatomical analysis of the main *C. longa* tissues (xylema, phloem, parenchyma and epidermis). All showed an appropriated pattern of development. At the end of the assay, the seedling was healthy and vigorous (Figure 1E). External epidermis tissue followed the pattern according to *Alpinia zerumbet* (Albuquerque and Neves, 2004). In parenchyma, the presence of idioblasts was observed with phenols. Thong et al. (2009) described the presence of tannin idioblasts in the basal regions of *Alpinia purpurata* seedlings. Albuquerque and Neves (2004) reported a great number of tannin idioblasts in the fundamental parenchymatic tissue of all studied vegetal parts in *A. zerumbet*. The same authors mentioned that occasionally tannins can be found in trichomes of the leaf edge, but trychomas were not observed in *C. longa* in the present study.

In monocots like *C. longa*, veins and bundles stretch along the leaf, forming parallel or striated nervation. Albuquerque and Neves (2004) observed that all secondary veins branch out from the main vein and grow parallel toward the edges in *A. zerumbet*. The conducting tissue, inserted in the parenchyma, has phloem facing

the abaxial face and xylem oriented towards the adaxial face of the leaf.

Xylem of *C. longa* was differentiated at shoot emergence, with the presence of numerous vein elements. Compared to other anatomical measurements, the xylem and phloem had less variation among the treatments (Figure 5D to E). This might have been because of the time that the plants were kept *in vitro* was not enough to realize a more pronounced differentiation of tissues. Obviously, the xylem differentiation process would be resumed, the slower the differentiation of xylem and phloem appears to occur at the time tested here. Another factor is due to the action of gibberellins which has a direct role in the differentiation of xylem; according to McKenzie and Deyholos (2011), the exogenous addition of the gibberellin GA_3 was essential to initiate thickening of xylem cells in *Linum usitatissimum* L along the cultivation of this species. The authors reported that the presence of GA_3 was essential to promote the differentiation process of this tissue as well as promoting thickness gains. In this work, no exogenous gibberellin was not added to confront the analyzed treatments.

Phase 2: Rooting phase

Although there was no cytokinin in the media for the rooting phase, SN responded to the supplemental auxin. The presence of a growth regulator seems to be necessary to increase SN in Zingiberaceae. Bharalee et al. (2005) did not observe any shoot growth from *Curcuma caesia* in base medium. The same was verified for *Z. officinale* by Kambaska and Santilata (2009) and for *C. amada* by Prakash et al. (2004). Recent studies have shown the role of auxins not only in cell elongation but also in allowing cells to progress through the G1/S transition of the cellular cycle (Perrot-Rechenmann, 2010).

In contrast, the longest root length (RL) values were seen in the control treatment, with no benefits seen with the addition of growth regulators. In this case, the amount of endogenous auxin may have been sufficient to stimulate cell elongation in the roots but not in the aerial part. This difference between the aerial part and roots may be due to the existence of at least five TIR1/AFB families of IAA receptors in the nucleus (Simon and Petrášek, 2011). It is likely that different receptors are active in the different parts of the plant (Vierstra, 2009). Villa et al. (2008) obtained similar results for *Rubus* species in which exogenous auxin did not improve root length total but did increase fresh matter of aerial part.

The results demonstrate that the auxin is important to provide mass increments *C. longa* root. The average total root dry mass was twice as high in the treatment 3 compared to treatment 2 and control. This is consistent with other studies demonstrating the effects of auxin in increasing root mass and number (Aslam et al., 2013; Aroonpong and Chang, 2015).

Conclusion

In the multiplication phase, the addition of BAP to the culture medium increased the fresh mass of the mother plant. The combination of auxin and cytokinins was vital for increasing the mass of the root system, and resulted in seedlings with a greater number of lateral roots.

DNA content varied in samples according to the treatments. When using *P. sativum* as an external reference standard, the content varied from 2.53 pg (in T5) to 2.38 pg (in T3), and when using *S. lycopersicum*, it ranged from 2.77 pg (in T4) to 2.62 pg (in T3).

There was quantitative variation in the measured anatomical characteristics. Epidermis and parenchyma were the tissues most affected by the action of regulators. In general, there was a thin layer of epidermis with rectangular cells, followed by parenchyma with octahedral cells. Xylem and phloem had already been differentiated with extensive leaf branching.

In the rooting phase, auxins were not necessary to increase length, but they were essential to increase root system mass and the presence of lateral roots.

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Micropropagation of *Launaea cornuta* - an important indigenous vegetable and medicinal plant

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An efficient micropropagation protocol was developed for the medicinal plant *Launaea cornuta* using green house axillary buds as explants. The best sterility was obtained at 30% (v/v) local bleach (JIK). Maximum shoot induction rate was achieved when axillary buds were cultured on Murashige and Skoog (MS) Media supplemented with 0.5 mg/L of 6-benzylaminopurine (BAP) for 3 weeks. The highest number of shoot multiplication was obtained when induced shoots were culture on MS media supplemented with 0.5 mg/L BAP and 0.2 mg/L NAA for 30 days. The best rooting response with regard to average root length, rooting percentage and number of roots was achieved within 4 weeks of culture of excised shoots on MS media having 0.5 mg/L BAP. Regenerated plants were successfully acclimatized and about 80 to 90% of plantlets survived under *ex vitro* conditions. About 170 plants were produced from a single nodal bud of *L. cornuta* after 60 days. A reproducible protocol was established for *in vitro* propagation of *L. cornuta*, an important indigenous vegetable with high medicinal value.

Key words: *Launaea cornuta,* tissue culture, micropropagation, axillary buds, tissue culture.

INTRODUCTION

Launaea cornuta commonly known as bitter lettuce or "mchunga" is a wild vegetable belonging in the family Asteraceae. It is indigenous to Kenya, Uganda, Malawi, Tanzania, Mozambique and Zimbabwe where it is mainly used as a vegetable (Jeffrey, 1966; Schippers, 2002), fodder for rabbits and sheep and to increase milk yield in cows (Burkill, 1985). It is rich in nutrients such as proteins, fat, carbohydrates, calcium, phosphorus, iron and ascorbic acid (Ndossi and Sreeramulu, 1991). It is exploited for its antidiabetic, anticancer, insecticidal (Kareru et al., 2007), antimalarial, antibacterial (Musila et al., 2013) and anthelmintic (Hiene and Heine, 1988) properties. It has been used to treat gonorrhea, syphilis, sore throats, coughs, typhus, nasal-pharyngeal infections, measles, swollen testicles, pain in the spleen, ear aches, hookworm eradication as well as fever (Hiene and Heine, 1988). *L. cornuta* is locally propagated vegetatively through rhizomes (Abukutsa, 2007). Development of a micropropagation protocol for *L. cornuta* can help to promote its cultivation and domestication which is currently limited to the coast of Kenya and countries bordered by the coast line (Abukutsa, 2007). The aim of

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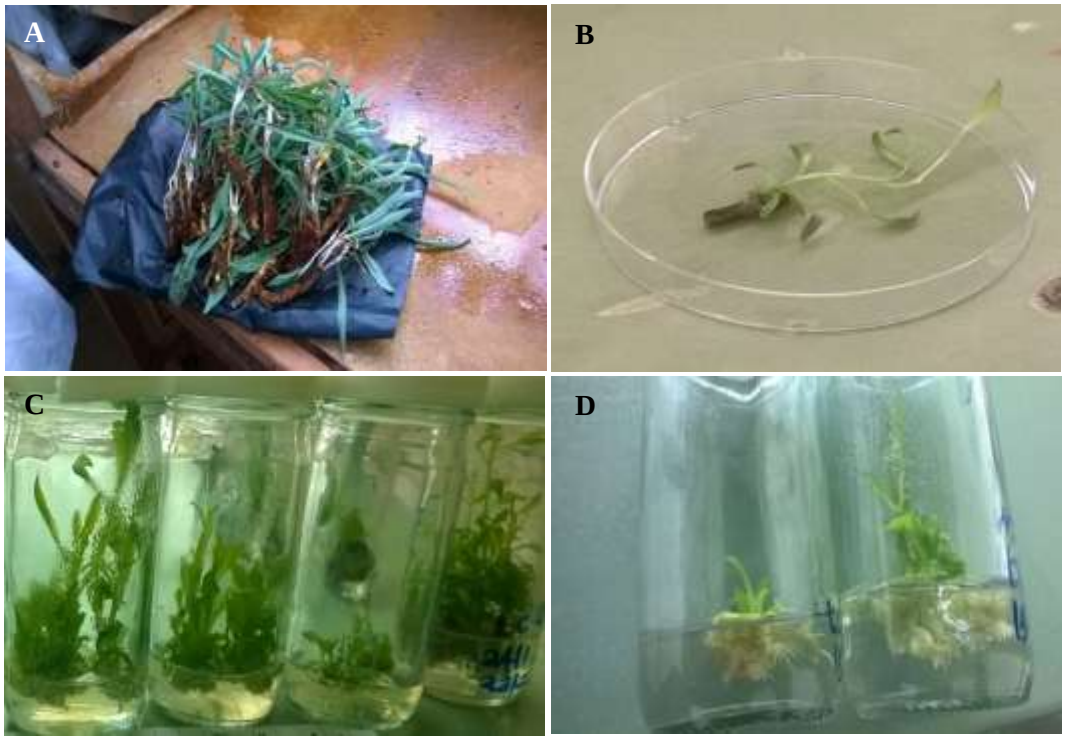

Figure 1. *In vitro* propagation of *Launaea cornuta.* A. *Launaea cornuta* plants with Rhizomes. B. Initiated shoot 4 weeks after culture on MS basal media with 0.5 mg/l BAP. C. Proliferated shoots 3 weeks after culture on MS basal media with 0.5 mg/l BAP and 0.2 mg/l NAA. D. Rooted shoots 2 weeks after culture on MS basal media with 0.5 mg/l IBA.

this study was to regenerate *L. cornuta in vitro* using nodal buds as explants. A micropropagation protocol is essential for rapid multiplication, utilization and conservation of *L. cornuta*.

MATERIALS AND METHODS

Establishment of plants in the green house and culture conditions

Launaea cornuta plants were collected from the field and planted in sterile soil in plastic bags and placed in the screen house at Kabete Biotechnology Center of Kenya Agricultural and Livestock Research Organization (KALRO). The plants were watered whenever necessary. All experiments were conducted using MS basal media (Murashige and Skoog, 1962) supplemented with 20 g/L sucrose and 3 g/L phytagel. The media was sterilised by autoclaving at 121°C for 20 min and dispensed equally into 10 ml universal bottles. Explants were cultured at a temperature of 24 ± 2°C and a photoperiod of 16/8 h light and dark conditions. Lighting conditions were provided by cool-white fluorescent lights of 3000 Lux.

Explant surface sterilization

Shoots from green house grown plants were cut just above the soil surface using clean scalpels and placed in bags (Figure 1). Shoots were defoliated in the tissue culture laboratory and individual 3 cm long shoot buds (explants) excised. Twenty one explants were placed into 5 different jam jars and rinsed 3 times with distilled water containing 3 drops of tween-20. This was followed by rinsing to remove the soap and addition of local bleach (JIK with 3.85% NaOCl) at 0, 5, 10, 20, and 30% into the jars for 20 min. The bleach was discarded and 70% ethanol added for 2 min. They were thoroughly rinsed with distilled water and held in 2% bleach solution awaiting culture. The dead tissues at the edges of the explant were cut off and the buds inoculated in MS media. The cultures were incubated under conditions described previously and the rate of explant survival and sterility for each bleach concentration recorded 30 days after culturing.

Shoot induction and proliferation

Explants were inoculated on the MS medium supplemented with BAP at 0, 0.1, 0.3, 0.5, 1.0 and 1.5 mg/L. The explants were cultured under conditions described previously and data on number of shoots per explants and shoot length recorded 3 weeks after culture. After 3 weeks of culture, the induced shoots were subcultured on MS media supplemented with 0.5 mg/L BAP in combination with NAA (0.1, 0.2, 0.3, 0.4, 0.5 mg/L). The cultures were incubated under growth conditions described previously. The shoots developed in the culture jars were maintained for 4 weeks and monitored for continuous elongation. Each NAA concentration was evaluated for shoot proliferation and growth.

Rooting of shoots and acclimatization

Shoots were subcultured into MS media containing IBA at 0, 01, 0.5

Table 1. Effects of different bleach concentrations on *Launaea cornuta* axillary bud surface sterilization.

Bleach concentration	Contamination rate	Survival percentage
40	0.000 ± 0.00[a]	1.000 ± 057[a]
30	0.000 ± 0.00[a]	3.0000 ± 0.57[b]
20	4.333 ± 0.67[b]	0.6667 ± 0.67[a]
10	4.667 ± 0.33[b]	0.3333 ± 0.33[a]
5	5.000 ± 0.00[b]	0.0000 ± 0.00[a]
0	5.000 ± 0.00[b]	0.0000 ± 0.00[a]

Each value represents the Mean±SE. Mean values followed by the same letter are not significantly different at P<0.05 (Duncan's Multiple Range Test).

Table 2. Effects of BAP on induction of shoots from axillary buds of *L. cornuta*.

BAP (mg/L)	Number of shoots per explant	Shoot length (cm)
0.0	4.667 ± 0.88[a]	0.867 ± 0.06[a]
0.1	6.667 ± 0.88[ab]	0.933 ± 0.03[a]
0.3	7.333 ± 0.88[abc]	1.367 ± 0.18[b]
0.5	11.000 ± 0.57[d]	1.967 ± 0.08[c]
1.0	10.333 ± 0.88[cd]	1.800 ± 0.11[c]
1.5	8.667 ± 0.66[bcd]	1.767 ± 0.08[c]

Each value represents the Mean±SE. Mean values followed by the same letter are not significantly different at P<0.05 (Duncan's multiple range test).

and 1 mg/L and maintained for 4 weeks under growth conditions described previously. The number of roots and root length was determined for each IBA concentration. Shoots with a well-developed root system were hardened in the glass house for 7 days by growing in sterile peat moss. Hardened plants were transplanted into soil and monitored until maturity.

RESULTS AND DISCUSSION

Sterilization of explants

Explant surface sterilization is mandatory and it serves to eliminate epiphytes and transient microorganisms. It is therefore important that the concentration of the sterilant be high enough to effectively kill all contaminants but low enough to avoid damaging the explants. To date, establishment of tissue culture for *L. cornuta* has not been reported. It is therefore not clear how the hollow stem sections of *L. cornuta* would respond to surface sterilization. To obtain the best sterilant concentration, we compared the effect of five different concentrations of NaOCl on sterility and shooting of nodal explants. The optimum concentration was 30% bleach, resulting in the highest sterility and number of shooting nodal explants (Table 1). At lower bleach concentrations the explants were greened. However, they did not shoot due to associated high rate of contamination. Bleach levels exceeding 30% caused scorching and eventual death of the explants (Table 1). The tissue available for isolation for microculture is an important factor that can influence the success rate of tissue culture. Vegetative tissues from plants growing outside have relatively high contamination making surface disinfection difficult. The ideal tissue is obtained from a small plant maintained in a relatively clean environment such as glass house. In this study, exposure to 30% of local bleach for 20 min was adequate to disinfect explant tissues obtained from glass house grown plants.

Shoot induction from axillary buds

Currently there are no reports on the *in vitro* response of any genotype of *L. cornuta*. To establish *in vitro* axillary shoot induction response of *L. cornuta* axillary buds, the effect of different BAP concentrations was compared. Multiple shoots were best induced on low BAP (0.5 mg/L), resulting in 11 shoots per axillary bud (Figure 1) after 3 weeks. Increasing the concentration of BAP to 1.5 mg/L resulted in a substantial reduction of shoot formation to 8.6 shoots per bud. Shoot length was significantly reduced when BAP level was reduced below 0.5 mg/L. However, increasing the BAP concentration beyond 0.5 led to statistically insignificant reduction (p>0.05) in shoot length (Table 2).

In many plant species, dormant axillary buds are

Table 3. Effects of different BAP: NAA combinations on proliferation of axillary shoots of *L. cornuta*.

BAP:NAA (mg/l)	Shooting rate (%)	Shoots per explant	Shoot length (cm)
0.5: 0.5	78^{d}	10.33 ± 0.88^{a}	1.967 ± 0.08^{a}
0.5: 0.4	89^{c}	14.33 ± 0.33^{b}	2.533 ± 0.06^{b}
0.5: 0.3	96^{ab}	16.33 ± 0.66^{b}	3.100 ± 0.1^{c}
0.5: 0.2	100^{a}	20.67 ± 0.66^{c}	4.567 ± 0.12^{d}
0.5: 0.1	100^{a}	19.00 ± 0.577^{c}	3.233 ± 0.14^{c}

Each value represents the Mean±SE. Mean values followed by the same letter are not significantly different at P<0.05 (Duncan's multiple range test).

induced to sprout into plants using cytokinins. For *L. cornuta*, presence of low BAP (0.5 mg/L) is paramount for efficient shoot induction. This is because shoots were produced at a significantly higher rate (p<0.05) for all media having BAP than for the control MS basal media lacking BAP (Table 2). New growth from nodal sections of *L. cornuta* was apparent in about 2 weeks of culture on media having 0.5 mg/L BAP. However, the new shoots elongated in 2 to 3 weeks. Emergence of new axillary buds (shoots) is dependent not only on explant type and hormone concentration, but also on other factors including hormone type, media composition, explant age and position.

Proliferation of axillary shoots

To test the ability of induced axillary shoots to proliferate, the effect of different BAP:NAA combinations on the number of shoots and shoot growth was evaluated. Media having BAP in combination with NAA gave an excellent shoot proliferation rate (Table 3). The highest number of shoot multiplication was obtained when media was supplemented with 0.5 mg/L BAP and 0.2 mg/L NAA (Figure 1). Favorable effects of these growth regulator combinations on shoot proliferation response has also been reported for several medicinal plants, such as, *Celastrus paniculatus* (Martin, 2006); *Coleus blumei* (Rani et al., 2006) and *Smilax zeylanica* L. (Sayeed and Roy, 2004). The regenerated shoots were healthy and attained a height of 4.6 cm within 4 to 6 weeks on media having 0.5 mg/L BAP and 0.2 mg/L NAA (Table 3). These shoots were normal and rootable.

Root formation and plant acclimatization

After six weeks of culture of elongated shoots on hormone-free MS basal medium, the rooting response of media with different concentration of IBA was tested on excised shoots. Roots formed on shoots within 4 weeks of culture on all media with IBA (Figure 1). However, the root number and length decreased with decreasing IBA concentration. The best rooting response with regard to the percentage of shoots producing roots (100%), the number of roots per shoot (7.3) and the average root length (3.3 cm) was achieved with 0.5 mg/L BAP (Table 4). Similar effects of IBA on root induction of shoots have been observed in other medicinal plants, such as *Solanum trilobatum* (Jawahar et al., 2004). *Cassia alata* (Hasan et al., 2008) and *Plumbaga zeylanica* (Chaplot et al., 2006). The rooted plants were hardened and transferred to soil where they grew normally with no morphological abnormalities**.**

Table 4. Effects of IBA on rooting of *L. cornuta*shoots.

IBA (mg/L)	Root length (cm)	Roots per shoot
0.0	0.967 ± 0.03^{a}	3.000 ± 0.57^{a}
0.1	1.200 ± 0.15^{a}	3.667 ± 0.66^{a}
0.3	2.067 ± 0.12^{b}	5.000 ± 0.80^{a}
0.5	3.267 ± 0.14^{c}	7.333 ± 0.57^{b}
1.0	2.400 ± 0.26b	4.667 ± 0.66^{a}

Each value represents the Mean±SE. Mean values followed by the same letter are not significantly different at P<0.05 (Duncan's multiple range test).

Conclusion

This study established for the first time a micro-propagation protocol for *L. cornuta* using axillary buds as explants. About 170 plants were produced from a single nodal bud after 60 days. The protocol described here can be used for rapid propagation, conservation of and exploitation of *L. cornuta* germplasm for their nutritional and medicinal value.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Seed origin, storage conditions, and gibberellic acid on *in vitro* germination of *Campomanesia adamantium* (Cambess.) O. Berg

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***Campomanesia adamantium* (Cambess.) O. Berg seeds (guavira) are recalcitrant and rapidly lose viability upon removal from the fruit, making difficult the long-term storage. *In vitro* germination could be used as an important tool to overcome the issues related to this short viability. It might help seed conservation and species propagation. The purpose of this study was to evaluate *in vitro* germination of guavira seeds collected from different sites and stored under different conditions. Also, the sowing of these seeds in MS medium supplemented with different concentrations of gibberellic acid (GA_3) was evaluated. Seeds from the local garden were treated with: 0, 1.0, 2.0, 3.0, or 4.0 mg L^{-1} GA_3. Seeds from a local farmer's market were treated with: 0, 2.5, 5.0, 7.5 or 10 mg L^{-1} GA_3. We evaluated the germination rate, the average length of the shoot and main root, and the number of leaves. The *C. adamantium* seeds were sown immediately after harvest and treated with GA_3, regardless concentration, increased germination rate by at least 10%, whereas 1.0 mg L^{-1} GA_3 resulted in 100% germination. The shoot length increased linearly with increasing concentration of the growth regulator. Different concentrations of GA_3 had no effect on the development of the main root and leaves. Seeds acquired from a local farmer's market showed lower germination rate than those sown immediately after harvesting, and did not differ in the rate of germination under different treatments with GA_3. Furthermore, around 25% of those seedlings had abnormal leaf morphology. *C. adamantium* seeds stored at 4°C and -20°C for 60 days did not germinate successfully, suggesting that seeds under cold storage conditions cannot be used for germplasm purposes.**

Key words: Guavira, Cerrado, Myrtaceae, temperature.

INTRODUCTION

Campomanesia adamantium (Cambess.) O. Berg (guavira) is a native species to Cerrado belonging to the myrtle family Myrtaceae. The fruit is rich in phenolics compounds, with great antioxidant activity (Giada and

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Mancini Filho, 2006) and due to its sweetness and aromatic flavor it offers good market acceptance (Vieira et al., 2010). In addition to the unique flavor and nutraceutical properties of its fruit, this species presents desirable agronomic traits such as a high fruit per plant ratio (Melchior et al., 2006) and high genetic variability, which are useful for cultivar development and genetic selection (Oliveira et al., 2011).

Given these traits, *C. adamantium* is a good prospect for commercial production (Vieira et al., 2010). However, *C. adamantium* is not presently cultivated in commercial orchards, so fruits are directly harvested from natural populations. Several factors have limited the expansion of commercial orchards, such as the limited natural range of this species, susceptibility to insects and diseases, and poor post-harvest preservation during transport and storage (Vieira et al., 2010). However, the greatest limitation is the plant (seedling) propagation due to the recalcitrance characteristics and low seed germination efficacy (Melchior et al., 2006; Scalon et al., 2012; Dresch et al., 2012). Therefore, basic researches on seed storage, germination, establishment conditions (Dresch et al., 2012), as well as the development of new and efficient asexual propagation techniques (Vieira et al., 2010) becomes critically important in order to produce seedlings, establishing a germplasm collection for preservation, and developing commercial production.

In vitro tissue culture has been successfully used for species preservation, seedling production, and selection of disease-resistant plants. *In vitro* propagation or micropropagation presents countless benefits, including rapid mass production (Moraes et al., 2007; Arrigoni-Blank et al., 2011), year-round production of uniform plants, increased biotic and abiotic stress resistance, elimination of phytosanitary problems (Moraes et al., 2007; Dias et al., 2011), and facilitation of germplasm maintenance and exchange (Braun et al., 2010). Field-grown or wild plants may not provide a suitable source for *in vitro* culture due to endogenous contamination (Soares et al., 2009). Whereas, healthy explants can be developed from vegetative material grown from seeds germinated *in vitro*. However, Oliveira et al. (2013) highlights that an embryo is the result of genetic recombination with a different genotype limiting the cloning process of superior individuals established in the field. *In vitro* germination and high quality seedling establishment (great vigor and phytossanitary conditions) may be used to initiate *in vitro* micropropagation. These techniques will assist in *C. adamantium* conservation and seedling production, avoiding the limitations imposed by propagation via seed.

In recalcitrant seeds, the lifetime is very limited, and to guarantee the species reproduction favorable environmental conditions for germination are required. In this sense, Dresch et al. (2012) found that the maintenance of high moisture content of *C. adamantium* seeds is essential. The same authors found that at 25°C, seeds submitted to drying for 16 h (27% water content) followed by storage for 18 days lose their germinating power (0%) in relation to the newly processed seeds with 57% moisture content (52%), evidencing the sensitivity to desiccation.

Seed germination is a complex process, controlled by both physical and internal regulating factors, and in this sense, GA plays very important role in controlling and promoting germination, stem elongation and meristmetic tissue development (Gupta and Chakrabarty, 2013). The application of gibberellic acid, although do not influence the recalcitrance of seeds, plays an important role in stem or internode elongation, stimulating cell division and expansion, promoting favorable conditions for the germination and seedlings establishment.

For all these reasons, our research objective was to evaluate the efficiency of *in vitro* germination of *C. adamantium* seeds collected from different sites, and, although the seeds are classified as recalcitrant, seed germination stored without desiccation was evaluated, maintaining the high moisture content, under different conditions and treated with different gibberellic acid concentrations.

MATERIALS AND METHODS

Fruits *C. adamantium* (Camb.) O. Berg were harvested in November 2013 from plants grown in the Garden of Medicinal Plants at the Faculdade de Ciências Agrárias, Universidade Federal da Grande Dourados, Mato Grosso do Sul (MS), Brazil. In November 2014, fruits were obtained from a farmer's market in the Dourados city (MS).

The fruits from plants grown in the garden were processed immediately after harvest, while the fruits from a farmer's market were processed after three days of harvest, which were kept by the farmer at favourable environmental conditions. The fruits were pulped and the seeds were separated under running water. In a laminar flow cabinet, seeds were rinsed with 70% ethanol, immersed in 2.5% sodium hypochlorite for 5 min, washed three times in sterilized water, and superficially dried with sterilized filter paper. After this procedure and in order to sterilize external surfaces seeds were used in one of five experiments designed under a completely randomized design. In the first two experiments, the initial germination capacity of the seeds was immediately evaluated. For the other experiments, the seeds were stored in brown paper bags for 60 days at one of the following temperatures: ±25°C (environmental conditions maintained by air conditioning), 4°C, or −20°C. After storage, seed surfaces were sterilized a second time as previously described. All experiments tested the effects of different concentrations of gibberellic acid (GA_3) on germination efficacy.

In the first experiment, seeds from the local garden were treated with different GA_3 concentrations: 0 (control), 1.0, 2.0, 3.0, or 4.0 mg L^{-1}. Each of the five treatments included four replicates of three culture flasks with seven seeds each. In the other experiments seeds from a local farmer's market were treated with different GA_3 concentrations: 0 (control), 2.5, 5.0, 7.5, or 10 mg L^{-1}. Each of the five GA_3 treatments included five replicates of one culture flask with five seeds each. In every experiment seeds were sownin 260 mL glass culture flasks containing 30 mL of MS culture medium (Murashige and Skoog, 1962), with 30 g L^{-1} of sucrose, 100 mg L^{-1} of myo-inositol, 6 g L^{-1} of agar, and the specified concentration of

Table 1. Analysis of variance (ANOVA) summary for the *in vitro* germination of *Campomanesia adamantium* seeds extracted from fruit collected in the local garden.

SV	DF	MS			
		G	ALS	ALMR	ALL
GA_3 concentration	4	0.13*	0.65^{ns}	0.14^{ns}	0.01^{ns}
Residue	12	0.03	0.29	0.10	0.02
VC (%)		11.6	12.3	17.3	7.9
OA		96.3	4.4	1.8	2.4

**, * and ns, significant at 1 and 5% and non-significance, respectively, by the test F. SV, Source of variation; DF, degrees of Freedom; SM, mean square; VC, variation coefficient; OA, overall average. Germination - G (%), Germination of normal plants (GNP) and abnormal (GAP); ALS, Average length of shoots (cm); ALMR, Average length of main root (cm); ALL, average number of leaves.

Table 2. Analysis of variance (ANOVA) summary for the *in vitro* germination of *C. adamantium* seeds extracted from fruit exposed at the local farmer's market.

SV	DF	SM					
		G	GNP	GAP	ALS	ALMR	ALL
GA_3 concentration	4	0.04^{ns}	0.16^{ns}	0.14^{ns}	0.17^{ns}	0.03^{ns}	0.48^{ns}
Residue	16	0.05	0.10	0.12	0.07	0.13	0.17
VC (%)		30.5	69.5	79.5	20.9	28.2	29.3
OA		49.6	26.1	24.3	1.2	1.2	1.8

**, * and ns, significant at 1 and 5%, and non-significance, respectively, by the test F. SV, Source of variation; DF, degrees of Freedom; SM, mean square; VC, variation coefficient; OA, overall average. Germination - G (%), Germination of normal plants (GNP) and abnormal (GAP); ALS, Average length of shoots (cm); ALMR, Average length of main root (cm); ALL, average number of leaves.

GA_3, with the final pH adjusted to 5.8. The medium was sterilized in an autoclave for 20 minutes at 121°C, under 1.5 atm of pressure.

After sowing, the flasks were transferred to a growth chamber set at 25 ± 2°C. Seeds from the garden were subjected to an initial 15-day period of darkness, while seeds from the market were kept in darkness for 7 days; after this period, all seeds were grown under light with a photosynthetic photon flux density of 45 µmol m^{-2} s^{-1} and a photoperiod of 14 h.

After day 45, seeds from the local garden fruit were scored for percent of germination, average length of the shoot (cm), average length of the main root (cm), and average number of leaves. After day 30, seeds from the farmer's market fruit were scored for germination rates (total = seeds that developed roots; normal = seedlings with root and leaf; abnormal = seedlings with stem axis but no leaves), average length of shoot (cm), average length of the main root (cm), and average number of leaves.

Percentage data were transformed to arcsine values, while count and continuous data were transformed to square root prior ANOVA analysis. Means were compared by polynomial regression using the statistical software package Winstat (Machado et al., 1999).

RESULTS AND DISCUSSION

According to the ANOVA different concentrations of gibberellic acid (GA_3) showed a significant effect (p=0.05) on *in vitro* germination of seeds collected from the garden and immediately inoculated after harvest (Table 1). Farmer's market fruit seeds did not show a significant response to different concentrations of GA_3 (Table 2).

Seeds stored at ambient temperature (approximately 25°C) germinated prematurely and became contaminated with fungi during storage, probably due to the combination of relatively high humidity and temperature, precluding their use in further experiments. The seeds stored at 4°C and −20°C for the period of 60 days lost their germinative power, with no germination observed 30 days after *in vitro* sowing. Our results corroborated those of Melchior et al. (2006) and Scalon et al. (2009), where the authors showed that guavira seeds presented recalcitrance, lost germinative power during storage, and only germinated successfully when obtained from fruits right after their harvest and pulping.

Garden seeds sown right after harvest presented significant differences in percent germination when treated with different concentrations of GA_3. The addition of GA_3 to the culture medium, regardless of concentration, increased the percent of germination by approximately 10% over the control. Maximum germination was observed at 2.48 mg L^{-1} GA_3 (calculated value) (100%) (Figure 1A). For the seeds from market fruits, percent of

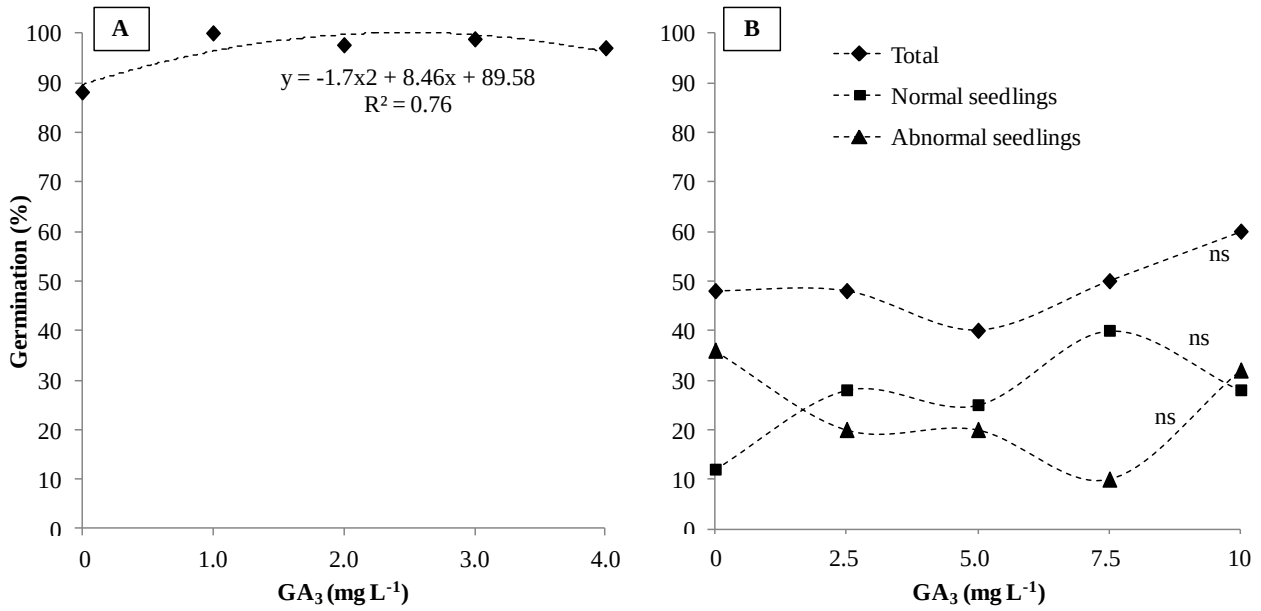

Figure 1. The effect of gibberellic acid (GA_3) in the MS medium on *in vitro* germination of *C. adamantium*. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

germination did not differ at different concentrations of GA_3 (Figure 1B).

In the first and second experiments, the seeds were sowing immediately after surface sterilization, but the garden and market seeds had different germination rates. These differences might be related to the amount of time between harvest and seed extraction. *C. adamantium* seeds are known to lose their viability when kept in the fruit, and they germinate most successfully right after harvest (Melchior et al., 2006). We could not establish a harvest date for the market fruits, but the seeds had already lost viability compared to those from the freshly harvested garden fruits. More research will be needed to identify the factors that lead to the reduction of viability.

Market seeds that were treated with 10 mg L^{-1} of GA_3 showed abnormal morphology in approximately 25% of the seedlings (Figure 1B), with the primary leaves atrophied or undeveloped. The development incomplete or abnormal of seedlings may be attributed to the fruit harvest time and storage conditions. The harvest date and storage conditions prior to selling might affect factors that are essential for proper seedling development.

In the polynomial regression using garden seeds, shoot length demonstrated a linear response to the concentration of GA_3 (Figure 2A). The long shoots (4.8 cm) grew in the culture medium with 4.0 mg L^{-1} of GA_3. In market seeds, different concentrations of GA_3 did not affect shoot length over the control; average seedling length was 1.2 cm (Figure 2B). Main root development was not influenced by gibberellic acid at any concentration in any of the experiments (Figure 2A and B). Soares et al. (2012) reported a positive influence of GA_3 on the shoot length in the orchid *Dendrobium nobile* Lindl. They also observed that plant height increased linearly with the increase of GA_3 concentration, suggesting a correlation between the two. According to Santos et al. (2013), gibberellin use may inhibit or minimize the impact of adverse factors in the quality and performance of seeds, and gibberellins increase the speed at which seeds emerge and aid in seedling development. During *in vitro* propagation, species that are sensitized by gibberellic acid elongate more rapidly and can be transferred from culture more quickly, allowing efficient production of large numbers of robust individual plants (Alcantara et al., 2014).

Simões et al. (2012) studied the effect of gibberellins, at the same concentrations we tested with the garden seeds, during *in vitro* germination of long pepper (*Piper hispidinervum* C. DC.), and contrary to our results, the authors found that shoot length responded negatively, with the longest shoots observed at the lowest concentration, 1.0 mg L^{-1} of GA_3. It is important to emphasize that growth regulators can affect cultivated species in different ways, and the classes, concentrations (Bastos et al., 2007), and the presence of endogenous phytoregulators can induce different responses in plants (Dias et al., 2008). Although *Eucalyptus dunnii* Maiden belongs to the same family (Myrtaceae) as *C. adamantium*, Navroski et al. (2013) did not find that GA_3 had a positive effect on the *in vitro* shoot elongation of this species. Treatment with GA_3 reduced germination and shoot length while increasing callus formation.

Species respond differently to gibberellins depending on tissue type, developmental stage, hormone

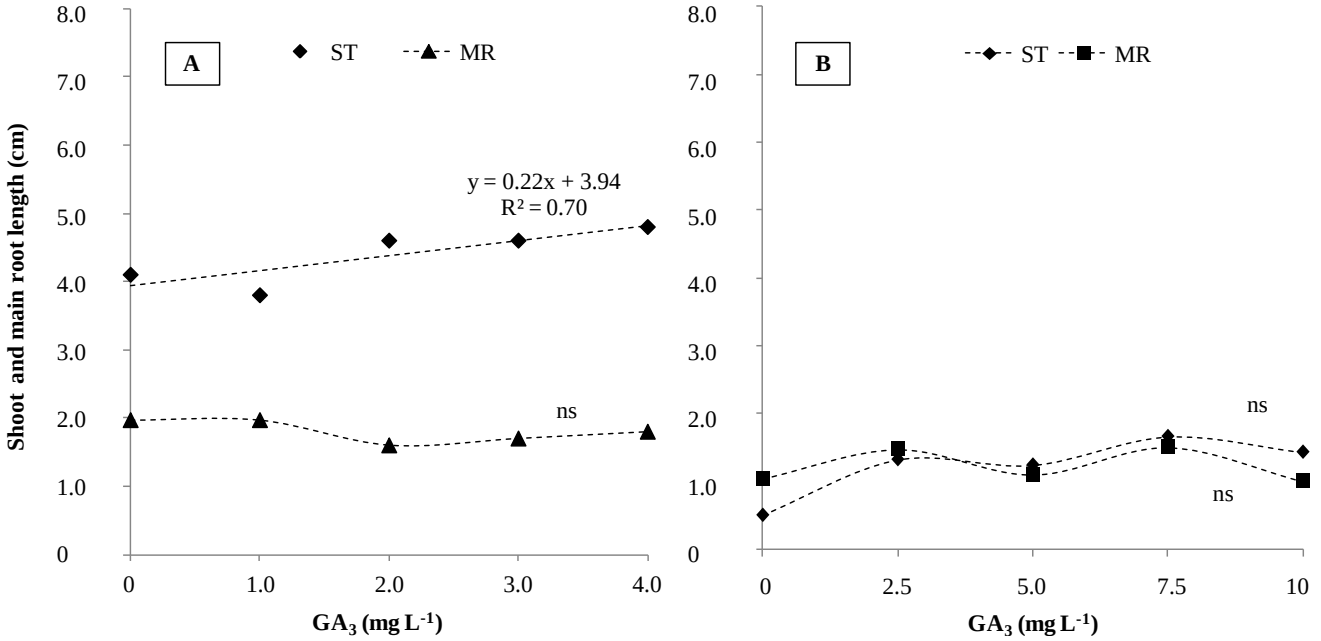

Figure 2. Average length of shoots (ST) and main root (MR) of *C. adamantium* seedlings germinated and *in vitro* cultivated in MS medium with different gibberellic acid (GA_3) concentrations. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

concentration, and interactions with endogenous factors. Different concentrations of GA_3 did not influence root growth, regardless of seed origin or storage treatment. Torres and Borges (2013) found similar results with *Capsicum frutescens* L. (chili pepper), with no significant difference in seedling root growth between the control and treatments with gibberellin. In contrast, Simões et al. (2012) found that the same concentrations of GA_3 that we used with the garden seeds reduced root length in long pepper (*Piper hispidinervum*) seedlings. However, according to Lima et al. (2009), in many species, including passion fruit, tangerine, soursop, and lemon trees, gibberellins promote cellular stretching and stimulate the primary root to break the tissues that restrict its growth.

We did not find a significant difference in the average number of leaves between seed types or among treatments (Figure 3A and B). However, seedlings from market seeds had a greater average number of leaves when germinated in the presence of GA_3 (Figure 3B). Machado et al. (2005) studied the effects of different concentrations of gibberellins during the acclimatization process in micropropagated rootstock of the apple cultivar Marubakaido and observed that the number of leaves was positively affected. They found that gibberellic acid induced the plants to produce a larger number of leaves by overcoming apical bud dormancy.

Garden seeds germinated right after harvest (Figure 4A) showed an increase in shoot length and leaf number in the presence of GA_3 compared to the control, although shoot length and number of leaves were not influenced by different concentrations of the regulator. Seedlings from market seeds (Figure 4B) that were germinated in a medium containing GA_3 showed better leaf blade developed, longer internodes, and a thicker main root when compared to the control.

Conclusion

The use of gibberellic acid, regardless concentration, promoted an increase of 10% in germination of seeds inoculated in a culture medium right after fruit harvest, however the use of 2.48 mg L^{-1} (calculated value) lead to 100% germination. Shoot length increased linearly as the growth regulator concentration increased. At the studied concentrations, GA_3 did not affect main root and leaf development. Seeds extracted from fruits at the farmer's market had lower a germination rate and did not respond to the different treatments with gibberellic acid. The seeds had an elevated percentage (±25%) of seedlings with abnormal leaf morphology. Seeds stored at 4°C and −20°C for 60 days did not germinate, whereas seeds stored at ambient temperature (±25°C) germinated prematurely. These storage conditions are not suitable for seed conservation.

Conflict of Interests

The authors have not declared any conflict of interests.

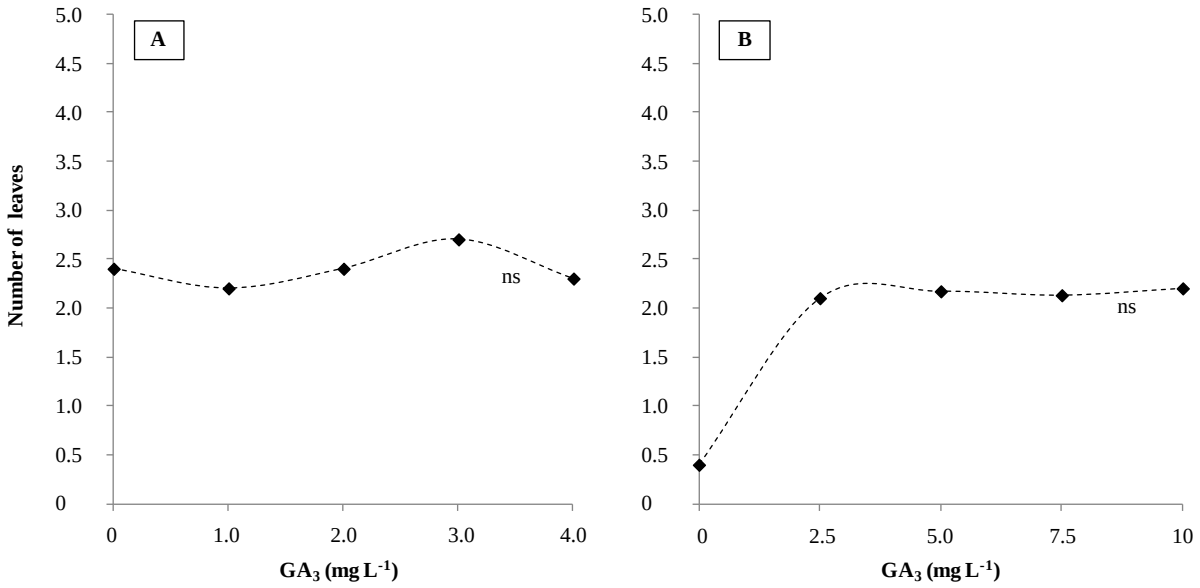

Figure 3. Average number of leaves of *C. adamantium*, seedlings germinated and *in vitro* cultivated in MS medium with different gibberellic acid (GA_3) concentrations. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

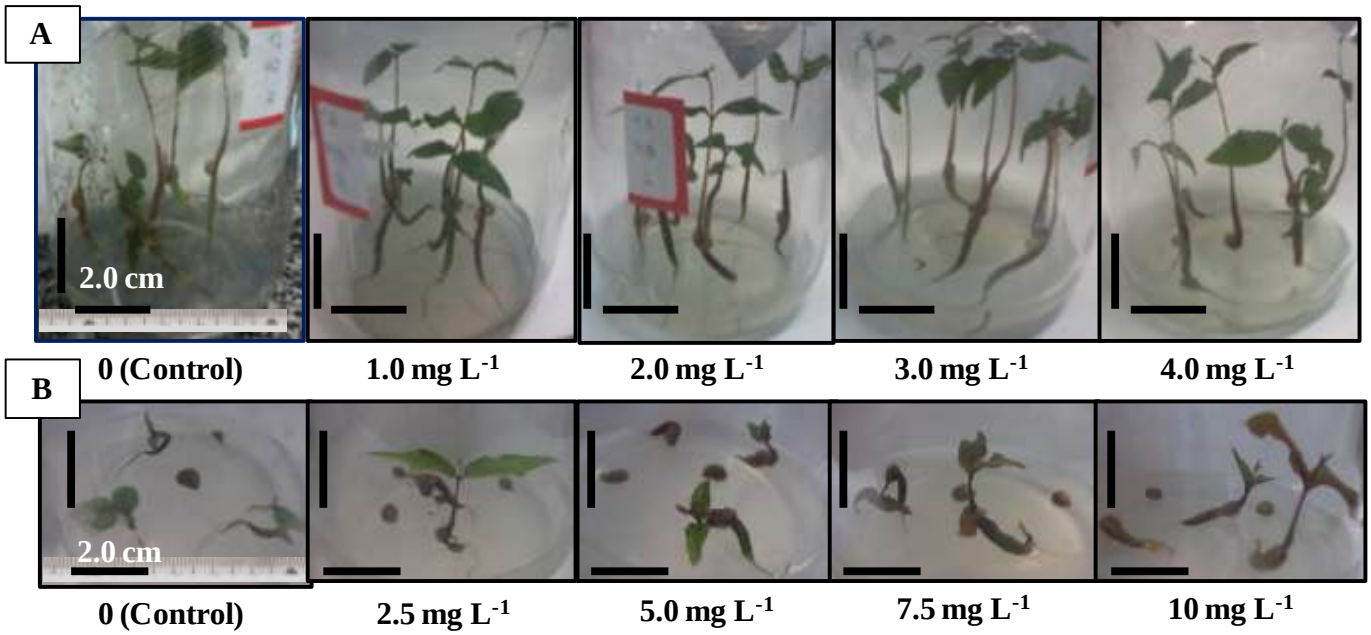

Figure 4. General appearance of *C. adamantium* seedlings germinated and *in vitro* cultivated in MS medium with different gibberellic acid (GA_3) concentrations. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

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Full Length Research Paper

Proteomic variation in Korean ginseng (*Panax ginseng* C.A. Meyer) isolates from different geographic regions

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Korean ginseng is a traditional medicine that is widely used in Korea. In this study, a proteomic approach was used to investigate variations in Korean ginseng isolates that are associated with ecologic and geographic differences. Ginseng samples were collected from four geographically isolated locations in Korea: North gyeonggi, Gochang, Geumsan and Kanghwa. Two-dimensional gel electrophoresis (2-DE) and peptide fingerprinting of tryptic digests by mass spectrometry (MALDI-TOF) revealed primary ginseng root region-specific variations in protein profiles in these distinct areas. Thirty seven (37) major proteins that are common to the main root of ginseng at all four geographic sites and six proteins that are specific to the main root of a local ginseng (Kanghwa) were identified. Most of the major common proteins identified could be classified into the following functional categories: (i) stress response; (ii) transcription and translation; (iii) nucleotide metabolism; (iv) plant hormone response; (v) signal transduction; (vi) protein degradation; (vii) protein destination and storage; and (viii) unassigned. The results show that Korean ginseng species can be distinguished on the basis of classical proteomics.

Key words: *Panax ginseng* C.A. Meyer, 2-DE, peptide fingerprinting, classical proteomics.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a traditional medicine for more than a thousand years in Korea to increase stamina and the capacity to cope with fatigue and physical stress. Ginseng has many reported health benefits, including regulation of blood sugar level and anti-stress, anti-cancer, anti-oxidant and anti-aging activities (Helms, 2004; Yoo et al., 2006; Koo et al., 2007). Korean ginseng is found to have such main properties as ginsenoside, polyacetylene, acid polysaccharide, anti-oxidative aromatic compound, and insulin-like acid peptides. The number of ginsenoside types contained in Korean ginseng (38 ginsenosides) is substantially more than that of ginsenoside types contained in American ginseng (19 ginsenosides). Furthermore, Korean ginseng has been identified to contain more main non-saponin compounds, phenol compounds, acid polysaccharides and polyethylene compounds than American ginseng and

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Figure 1. Locations of ginseng plantations from which the ginseng was collected in this study.

Sanchi ginseng (Choi, 2008). Vast quantities of ginseng are consumed in Korea, where the ginseng trade is an important business. Traditionally, the authentication of Korean ginseng sites of origin has relied on morphologic inspection (Hong et al., 2012). In many cases, this approach is unreliable because the roots of different Korean ginseng isolates are often morphologically similar. Thus, a more quantitative analysis of Korean ginseng sites of origin may be a useful reference tool for promoting fair trade of ginseng in the Korean herbal industry. Recently, high performance liquid chromatography (HPLC) separation of different ginsenosides (Fuzzati et al., 1999; Lee and Marderosian 1981; Li et al., 2000) and amplification of polymorphic DNA (Tochika-Komatsu et al., 2001; Um et al., 2001; Mihalov et al., 2000) have been used to screen various types of ginseng. However, as a tool for distinguishing ginseng isolates with different origins, these approaches have several limitations, including reproducibility. A potentially efficient and reliable alternative for characterizing regional ginseng isolates would be a proteomic approach.

The proteome is the entire complement of proteins expressed by a genome in a cell, tissue or organism. More specifically, it is the set of proteins expressed at a given time under defined conditions. Recent technical improvements in two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) have made it possible to compare hundreds of proteins and identify patterns of differential protein expression, thus providing a framework for understanding target tissue function. Here, a proteomic approach was used to investigate natural variations in total protein profiles among Korea ginseng isolates from four different geographic regions. A proteomic approach for comparing within-species variations among Korean ginsengs could be a useful separation system for resolving questions of sites of origin and distinguishing different ginseng subspecies.

MATERIALS AND METHODS

Fresh 6-year-old Korean ginsengs were collected from four well-known Korean ginseng cultivation regions (North gyeonggi, Kanghwa, Geumsan, and Gochang) in Korea: North gyeonggi (between 37°53′11″N 127°11′25″E to 37°54′26″N 127°12′39″E), Kanghwa (between 37°44′46″N 126°30′03″E to 37°54′26″N 126°30′43″E), Geumsan (between 36°00′45″N 127°30′38″E to 36°06′19″N 127°31′48″E), and Gochang (between 35°48′07″N 127°25′50″E to 35°49′52″N 127°26′45″E) (Figure 1). The Northern regions (that is, North gyeonggi, Kanghwa) were colder than the southern regions (that is, Gochang, Geumsan). The Geumsan region showed the highest percentage of the days (25.02%), which was about a quarter of a year. North gyeonggi and Kanghwa showed a percentage of approximately 22 to 24%. The Gochang region showed that the lowest days with a peak air temperature above 30°C was counted.

Sample preparation

Ginseng samples were stored at 4°C until protein extraction. The

main roots (body) of the ginseng plants were cut and weighed before being ground in extraction buffer. Samples (10 g) were ground in a mortar with liquid nitrogen and incubated with sample buffer (0.3% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl pH8.0, 200 mM DTT) at 100°C for 10 min. The solution was transferred to ice and incubated with sample buffer II (DNase I, RNase A, 50 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$) for 10 min. After centrifugation at 15,000 × g for 30 min, supernatants were collected and precipitated with 10% trichloroacetic acid (TCA) solution overnight at -20°C. Protein pellets were washed with ice-cold acetone at least three times to remove contaminants and solubilized in a solution containing 8 M urea, 2 M thiourea, 100 mM DTT and 4% (w/v) CHAPS. Protein concentrations in samples were determined using the 2-D Quant protein assay kit (Amersham Biosciences).

Two-dimensional electrophoresis and image analysis

For 2-DE gels, samples were diluted into isoelectric focusing (IEF) buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.8% carrier ampholytes (pH 4.0 to 7.0 and pH 3-10 NL) and a trace of bromophenol blue to yield the desired protein amount in a volume that could be adsorbed by the immobilized pH gradient (IPG) strip. These diluted samples were used to rehydrate 11 cm IPGs for 12 h at 50 V. All IEFs were performed using the Protean IEF Cell (Bio-Rad) with an 11 cm IEF tray. After rehydration, the following voltage program was applied to the IPG strips: a linear ramp to 250 V over 15 min, followed by a linear ramp to 8,000 V over 2.5 h and then a constant 8,000 V for 4.3 h, for a total of 44,000 Vh. For cup-loading gels, IPG strips were passively rehydrated overnight in IEF buffer and the rehydrated strips were placed gel-side up in a cup-loading tray. A set of cups was placed 1 cm away from the anode end. A 65 µg sample was loaded into the cups, and movable electrodes were placed on both ends of the strips. The strips were focused according to the following protocol: 500 V for 5 min, 4,000 V for 1.5 h, a linear ramp to 8,000 V for 3 h, and 8,000 V for 20,000 Vh. Focused IPG strips were stored at -80°C before equilibration and separation in the second dimension by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. After IEF, IPG strips were equilibrated by immersion in 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 30 mM DTT for 10 min, followed by immersion in 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 5% iodoacetamide for 10 min. The IPG strips were then placed on top of 10% polyacrylamide gels and embedded in hot 0.5% agarose (about 70°C) containing bromophenol blue. Separation was performed at a 80 mA constant current with external cooling until the tracking dye migrated to within 1 cm of the bottom of the gel. Upon completion of 2-DE SDS-PAGE, gels were stained with SilverQuest (Invitrogen, Carlsbad, CA, USA) stain as directed by the manufacturer. All experiments were performed in triplicate, and the representative single gel images represented in the figure. Spot detection and analysis was performed using the PDQuest version 8.0.1 software (Bio-Rad).

MALDI-TOF mass spectrometric analysis and protein identification

For protein identification, spots were excised from the gels and subjected to *in situ* digestion with trypsin as described previously (Savijoki et al., 2005). The digested supernatant fluid was mixed with MALDI matrix (α-cyano-4-hydroxycinamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) and spotted onto an MTB AnchorChip TM 600/384 MALDI plate (Bruker Daltonik), and peptide masses were determined using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonik). Calibration was carried out based on the internal mass of trypsin. Peptide masses were matched with the theoretical peptides of plant proteins in the National Center for Biotechnology Information (NCBI) database, using the MASCOT software and the MS-Fit software of Protein Prospector (website: http//prospector.ucsf.edu). The peptide mass fingerprint search included a few plants (*Arabidopsis thaliana* and *Oriza sativa*).

RESULTS AND DISCUSSION

Protein maps resolution and analysis

An optimization of 2D-PAGE protocol was used to obtain sharp protein maps from 4 origins of *Panax ginseng*. A side-by-side comparison of 2-DE gels of 65 µg samples of ginsengs from four different geographic regions was prepared by in-gel rehydration (Figure 2 *upper panel*) or cup loading (Figure 2 *lower panel*). Protein precipitation can be clearly seen as a collapsed line of unresolved spots in the in-gel rehydration sample; this was not observed when the sample was cup loaded. More proteins were present in the pH 4 to 7 range in the cup-loaded gel, and proteins were better resolved, with less horizontal streaking and improved spot quantification.

Protein spot identification

Three replicates of 4 origins of *Panax ginseng* were run on 2-DE. Artifacts or protein spots that could not be confidently verified as true matches, were disregarded rather than manually edited. Cut-off values for which 95% of observed data were valid was determined. An analysis of individual ginseng samples collected from different geographic regions in Korea by 2-DE showed clear proteomic variations, revealing both region-specific proteomic similarities and differences among ginseng samples. The total number of main root protein spots in ginsengs grown at North gyeonggi, Gochang, Geumsan and Kwanghwa was 400, 580, 313 and 414, respectively (Figure 3). A comparison of ginseng protein patterns obtained from the four different geographic regions revealed that 165 protein spots were common to all four regions. Small amounts of total proteins with masses ~17 to 40 kDa and pI values ~5.0 to 6.6 were detected in ginseng main roots collected from North gyeonggi and Geumsan, whereas those from Gochang and Kwanghwa contained high amounts of these proteins (Figure 3). This 2-DE analysis also clearly showed that the proteome pattern for ginseng main roots collected in Kwanghwa was distinct from those of ginsengs collected from North gyeonggi, Gochang and Geumsan. These differences in the Kwanghwa ginseng proteome may reflect genetic diversity caused by geographic isolation and time, as well as by variations in local environmental conditions and breeding cycles.

Kwanghwa region has a profitable environmental condition such as soil and regional climate for the

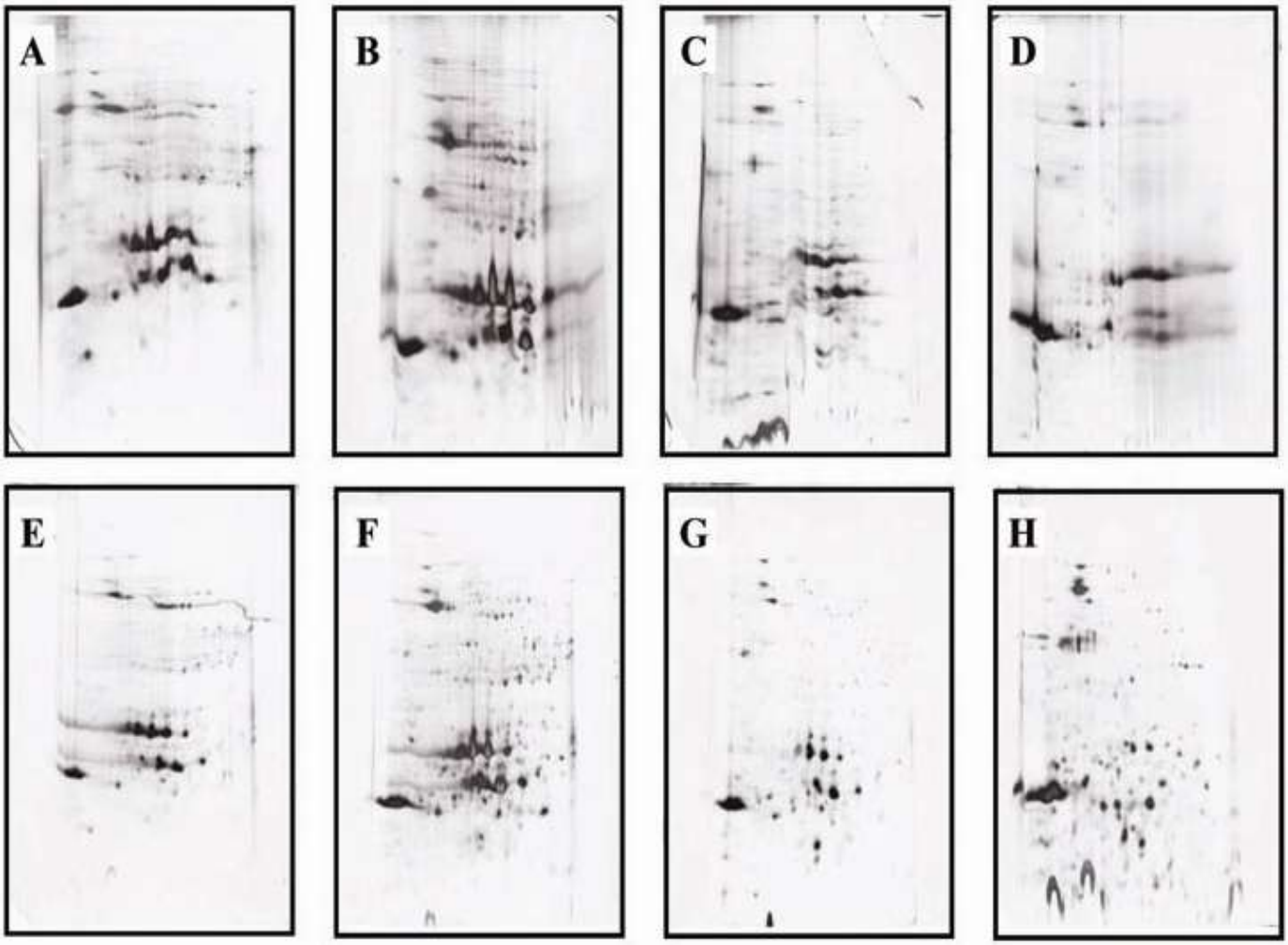

Figure 2. In-gel rehydration loading compared to the cup-loading technique. Representative 2-DE gels of different ginsengs (65 µg/sample) from four different geographic regions. *Upper panel:* pH 4 to 7 IPGs loaded using in-gel rehydration; *lower panel:* cup-loaded pH 4 to 7 IPGs. (A, E) North gyeonggi; (B, F) Gochang; (C,G) Geumsan; (D,H) Kwanghwa.

ginseng species. The climate of Kwanghwa is characterized by the relatively low daily temperature and large diurnal variation with plenty of solar radiation, long sunshine duration and less cloudiness. Because of the sea surrounding Kwanghwa island with low salinity and moderate wind, the salt contained in sea breeze is relatively low compared to other regions. It is also found that moderately coarse texture or fine loamy soils known as good for water drainage and for the growth and cultivation of the 'Kwanghwa-ginseng' are distributed throughout the areas around mountainous districts in Kwanghwa. A lower summer air temperature contributes to xylem compaction and hardness of ginseng, and in consequence, enriching crude saponin content and helping growth of ginseng. The implication of these differences is that there has been selective pressure on the Kwanghwa ginseng isolate to produce a particular proteome.

Thirty-seven protein spots (indicated in Figure 3) from among the 165 proteins common to all four regions were excised from 2-DE gels and digested with trypsin. Following extraction, the tryptic peptides were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The results are summarized in Table 1. A literature search revealed that the identified proteins could be categorized into the following functional categories: stress-responses (two proteins), transcription and translation (four proteins), nucleotide metabolism (one protein), plant hormone response (one protein), signal transduction (three proteins), protein degradation (one protein), and protein destination and storage (one protein). There were 13 proteins of unknown function and 11 unidentified proteins.

Among the differentially expressed proteins, the most highly represented categories were stress-response and transcription and translation. Two spots (spots 6 and 30)

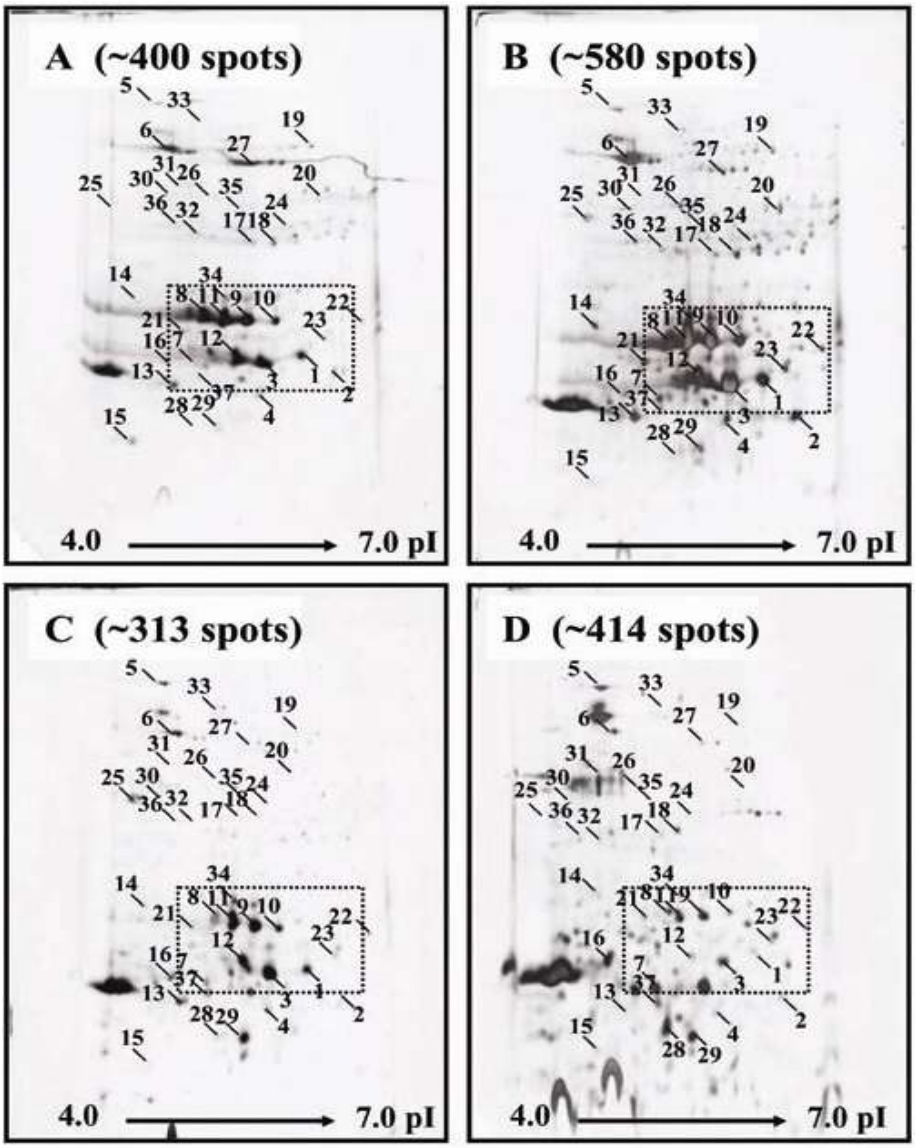

Figure 3. Typical 2-DE profiles of the main root of ginseng from four different geographic regions obtained using the cup-loading technique. (A) North gyeonggi; (B) Gochang; (C) Geumsan; (D) Kanghwa. All gels were loaded with 65 μg ginseng proteins and separated in the second dimension by SDS-PAGE using 10% (w/v) Tris-Tricine SDS gels, followed by silver staining. Protein spots with masses ~17 to 40 kDa and pI values ~5.0 to 6.0 that were differentially expressed among regional ginsengs isolates are boxed. Spots numbered 1 to 37 represent proteins exhibiting geographic region-specific differential expression. These proteins are listed in Table 1.

that corresponded to heat-shock proteins and four spots (spots 3, 4, 12 and 37) that corresponded to transcription and translation were identified. The most intense spot (spot 6 in Figure 3) was identified as heat-shock protein 70 (Hsp70), consistent with the reported prominent role of Hsp70 in ginseng root growth (Sung et al., 2001). This protein is also involved in cell rescue and defense against a number of environmental stress conditions, including heat, cold and drought, as well as chemical and other stresses (Guy and Li, 1998; Lin et al., 2001). Two major spots (spots 3 and 12) are transcription-related proteins that are thought to play a role in regulating ginseng responses to environmental stress. In *Arabidopsis*, transcription factor proteins are induced or repressed under different stress conditions, indicating a role in plant stress responses (Shinozaki et al., 2000). Histidyl-tRNA synthetase (spot 10), a protein involved in nucleotide metabolism, was also observed. In addition to its role in

Table 1. Characteristics of the 37 proteins consistently present in ginseng samples from four different ginseng's sites of area.

Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Stress response	6	cpHSC70-2 (Heat shok protein 70-7): ATP binding	5.0	77.06	gi | 15240578	27.4
	30	High molecular weight heat shock protein	5.0	71.57	gi | 6969976	32.8
Transcription and translation	3	ANAC087: transcription factor [*Arabidopsis thaliana*]	5.7	38.45	gi | 42573407	29
	4	ZCW32: DNA binding/transcription factor [*A. thaliana*]	6.2	29.03	gi | 18406408	28
	12	Protein: ORG2; DNA binding/transcription factor [*A. thaliana*]	5.63	28.76	gi | 152301	30
	37	Transcription factor [*A. thaliana*]	6.22	33.97	gi | 15221262	20
Nucleotide metabolism	10	Putative histidyl tRNA synthetase [*A. thaliana*]	5.71	21.98	gi | 110739016	58
Plant hormone response	35	S-adenosyl-L-homocystein hydrolase	5.8	54.05	gi | 71000473	33.1
Signal transduction	8	Putative protein kinase [*A. thaliana*]	5.74	66.00	gi | 9802793	21
	18	Protein serine/threonine kinase-like protein [*A. thaliana*]	5.69	67.78	gi | 8953410	25
	26	Kinase [*A. thaliana*]	79.5	6.10	gi | 22329045	20
Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Protein degradation	29	Hydrolase [*A. thaliana*]	5.27	23.78	gi | 15221055	56
Protein destination and storage	14	Endopeptidase/peptidase/threonine endopeptidase [*A. thaliana*]	4.70	26.05	gi | 15231824	37
Unassigned	1	Unknown protein [*A. thaliana*]	6.04	21.09	gi | 79479073	32
	2	Unknown protein [*A. thaliana*]	5.39	12.73	gi | 8404455	64
	3	Unknown protein [*A. thaliana*]	4.85	84.25	gi | 18402909	58
	9	Os02g0821900:Putative uncharacterized protein [*Oryza sativa* (japonica cultivar-group)]	5.77	28.40	gi | 110743760	43
	11	Os02g0821900 [*O. sativa* (japonica cultivar-group)]	5.43	32.27	gi | 115449795	44
	13	Putative protein (fragment) [*A. thaliana*]	4.12	11.95	gi | 5262206	74
	16	Hypothetical protein [*O. sativa* (japonica cultivar-group)]	5.98	22.18	gi | 54291450	66
	20	TH65 protein [*A. thaliana*]	6.48	73.60	gi | 110741724	15
	22	mRNA cleavage factor subunit-like protein [*A. thaliana*]	7.64	21.48	gi | 4914406	41
	24	Sulfotransferase [*A. thaliana*]	5.97	37.60	gi | 15230602	30
	27	Unknown protein [*O. sativa* (japonica cultivar-group)]	6.04	53.29	gi | 56783943	75
Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Unassigned	28	Unknown protein [*A. thaliana*]	5.72	27.95	gi | 15221706	39
	34	Protein phosphatase 2C-like protein [*O. sativa* (japonica cultivar-group)]	4.88	24.40	gi | 42409501	41

Table 2. Characteristics of the six proteins differentially expressed in ginseng samples from Kwangwha

Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Transcription	2	Transcription factor [*A. thaliana*]	6.22	33.97	gi | 15221262	20
	6	ANAC087 [*A. thaliana*]	5.70	38.44	gi | 42573407	33
Unassigned	1	Hypothetical protein [*O. sativa* (japonica cultivar-group)]	5.98	22.18	gi | 54291450	66
	3	Os10g0486900 [*O. sativa* (japonica cultivar-group)]	4.66	26.13	gi | 115482622	55
	4	Rid2 protein [*O. sativa* (japonica cultivar-group)]	5.13	30.99	gi | 77539080	69
	5	Unknown protein [*A. thaliana*]	5.72	27.95	gi | 15221706	39

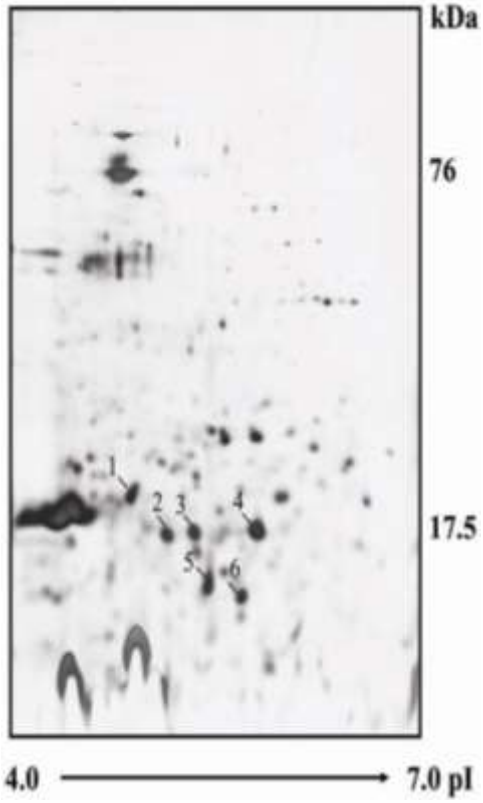

Figure 4. 2-DE of the main root from Kwanghwa ginseng. Note that protein spots 1 to 6 were common to all ginseng sample 2-DEs; however, these spots were significantly over-expressed in Kwanghwa ginseng. These proteins are listed in Table 2.

deciphering the genetic code during protein synthesis, this protein is reported to function in many other cellular processes that lead to stress responses, apoptosis and embryo development (Szymanski et al., 2000). In addition, S-adenosyl-L-homocysteine hydrolase (spot 35) was also identified, which is involved in plant hormone responses. This enzyme is responsible for maintaining active methylation for multiple lipid, protein and nucleic acid metabolic pathways (Tanaka et al., 1996). Several enzymes (spots 8, 18 and 26) involved in regulating a variety of cell functions, including proliferation, gene expression, cell-cycle progression, differentiation, cytoskeletal organization, cell migration and apoptosis, were also expressed; these proteins may be related to signal transduction. Further, a glycoside hydrolase (spot 29) was identified. The hydrolases are involved in degradation of glycoproteins and starch, and have various functions in plant defense and signaling (Minic and Jouanin, 2006). Interestingly, an endopeptidase (spot 14) was observed, which is involved in protein targeting and storage. This enzyme plays a key role in proteolytic processes that are associated with plant programmed cell death (Beers et al., 2000).

Finally, 13 proteins lacking good functional annotations were observed. The proteomic analysis further revealed six proteins that were specifically over-expressed in Kwanghwa ginseng (Figure 4 and Table 2). Two identified proteins (spots 2 and 6) are involved in transcription and three are unknown proteins.

Conclusion

In this study, a proteomic approach was used to analyze the protein expression profiles of ginsengs collected from four different regions of Korea: North gyeonggi, Gochang, Geumsan and Kanghwa. The proteomes of the different Korean ginseng isolates were different and could be used as distinguishing factors. The results presented here clearly showed intra-specific differences in the protein composition of ginseng plants collected from the different geographic regions. This probably reflects innate individual variation in protein synthesis, because genetic variation may be caused by local environmental conditions, geographic separation, nutritional status and time. Several common and region-specific protein spots were also identified in the 2-DE maps of different ginseng isolates. Ginseng proteomic data can be used as reference maps for comparative analysis of 2-DEs of

ginseng from different geographic regions.

Conflict of interest

The authors have not declared any conflict of interest.

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