EFFECT OF THIDIAZURON, NAA AND BAP ON *IN VITRO* PROPAGATION OF *ALSTROEMERIA AURANTIACA* CV. 'ROSITA' FROM SHOOT TIP EXPLANTS

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ABSTRACT
The objective of the study was to evaluate the potency of Thidiazuron (TDZ) as a plant growth regulator when compared to combined auxin (NAA) and cytokinin (BAP) in evoking morphogenic responses from *Alstroemeria aurantiaca* cv. 'Rosita' shoot tip explants. Shoot tips cultured on basal medium devoid of any plant growth regulators (PGRs) only increased slightly in length and formed only one leaf per shoot during the culture period. The addition of various PGRs to the induction or culture medium significantly influenced the number and length of shoots as well as the number of leaves formed. While low concentrations of TDZ (0.1 ¼M) had no significant effect and high concentrations (5.0 ¼M) were inhibitory, medium concentrations (0.4-1.0 ¼M) significantly increased the number and length of shoots as well as the number of leaves formed from the explants. The longest shoots were formed from explants cultured in media supplemented with 1.0 ¼M TDZ. Slightly better but comparable responses were observed from explants cultured on media supplemented with 1.0 mg/L BAP and low concentrations (0.01 mg/L) of NAA. The explants cultured in 1.0 mg/L BAP + 0.01 mg/L NAA formed the greatest number of shoots while those cultured in 1.0 ¼M TDZ formed the greatest number of leaves/ ex-plant. Increasing the NAA concentration to 0.1 mg/L and combining this with either 1.0 mg/L BAP or 1.0 ¼M TDZ depressed shoot formation and shoot length. In conclusion, TDZ at concentrations between 0.4 and 1.0 ¼M were just as effective as combined auxins (NAA) and cytokinin (BAP) in evoking morphogenic responses from *Alstroemeria aurantiaca* cv. 'Rosita' shoot tip explants.

Key words: Thidiazuron*, Alstroemeria aurantiaca,* explants

1.0 INTRODUCTION

Alstroemeria is among the top 6 most important cut flowers grown for the export market by Kenyan farmers, contributing over Kshs. 204.2 million compared to Roses (21.2 billion), Carnations (1.244 billion), Lilies (904.7 million), Hypericum (869.2 million) and Gypsophila (314.6 million) in a 39.8 billion shilling industry (HCDA, 2008). *Alstroemeria* has gained worldwide importance as a cut flower due to its high productivity, ease of management as an outdoor crop, high yield and the excellent vase-life of its attractive flowers available in a wide range of colors (Healy and Wilkins, 1979; Chepkairor and Waithaka, 1988; Bloom and Piott, 1990). Most of the *Alstroemeria* varieties are sterile hybrids, and are therefore commonly propagated by the division of rhizomes with attached roots (Healy and Wilkins, 1979). However, due to its slow rate of multiplication and high incidence of disease transmissions in conventional propagation practices, the development of a micro-propagation system is desirable. In Kenya, the potential yields and quality of *Alstroemeria,* as a cut flower, has not been realised due mainly to the unavailability of clean planting material for propagation (HCDA, 2008). *Alstroemeria* is susceptible to a number of viruses such as potyviruses and tomato spotted wilt virus as well as to fungal infections such as root rot and Botrytis (Bridgen *et al*. 1992). The alternative of importing clean plant materials, patented, and therefore attracting royalty payments, has proven prohibitive for most farmers (HCDA, 2008). Tissue culture technique, a powerful tool for mass propagation of clean material of desired genotypes, has been employed to develop protocols for rapid in vitro propagation of many clones and cultivars.

Early attempts to develop tissue culture propagation protocols were unsuccessful as *Alstroemeria* was viewed as a recalcitrant species together with other monocotyledonous plants. The culture, *in vitro*, of ovary tissues resulted in the development of non-morphogenic callus (Ziv *et al*. 1973). Low regeneration frequency (4%) was reported by Gonzalez-Benito and Alderson (1992) who succeeded in whole plant regeneration from mature embryos of a diploid cultivar 'Butterfly' via organogenesis. Whole plant regeneration was reported from mature embryos of a tetraploid cultivar via embryogenic callus (Hutchinson *et al*. 1994). In addition, *in vitro* multiplication of rhizomes and shoot tip explants has been demonstrated using a combination of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) in the culture medium (Gabryszewska, 1995). All the protocols mentioned above involved morphogenic control by using a combined auxin and cytokinin in the media.

Thidiazuron, (N'-phenyl-N'-1,2,3-thidiazol-5-ylurea, TDZ) is a phenyl urea that has gained importance as a potent plant growth regulator (PGR) for *in vitro* propagation systems of various crops (Fiola *et al*. 1990; Visser *et al*. 1992; Murthy *et al*. 1995; Hutchinson *et al*. 1996a; b). Despite its high efficacy in inducing morphogenic responses in vitro, there are limited reports where TDZ has been used to regenerate *Alstroemeria*. Earlier attempts to use TDZ alone to regenerate plants from mature ovaries of a tetraploid *Alstroemeria* was unsuccessful although combining TDZ (0.5 ¼M) and BAP (8.0 ¼M) induced multiple shoots from callus induced from mature zygotic embryos of a tetraploid *Alstroemeria* cultivar (*A. pelegrina* x *A. psittacina)* without an intervening callus phase (Hutchinson *et al*. 1994). Lin *et al*. (1997) reported direct shoot regeneration from excised leaf explants cultured *in vitro* on an induction medium supplemented with 10.0 ¼M TDZ combined with 0.5 ¼M IBA and regenerated on a medium supplemented with 2.2 ¼M BAP. To our knowledge, there are no reports of studies on TDZ-mediated regeneration of shoot tip explants of *Alstroemeria aurantiaca* cv. 'Rosita', a popular cultivar among Kenyan flower exporters. The main objective of the present study

was to compare the potency of TDZ with traditional auxin (NAA) and cytokinin (BAP) combination in the plant regeneration from *Alstroemeria aurantiaca* cv. 'Rosita' shoot tip explants.

2.0 MATERIALS AND METHODS

2.1 Stock Plants

Alstroemeria Aurantiaca cv. 'Rosita' stock plants grown under optimum cultural conditions were obtained from the Kenya Agricultural Research Institute (KARI) farm at Tigoni, Limuru. Limuru is at an altitude of 1800-2100 m above sea level.

2.2 Preparation of Explant and Sterilisation

Rhizomes from 3 month-old plants cleaned with detergent (bioagent) and rinsed in running tap water for 15 minutes. Excised shoot tips (1-2cm long) from the rhizomes were immersed in 95% alcohol for 5 minutes and subsequently rinsed in sterile distilled water for 3 minutes. The tips were then placed in 0.5% sodium hypochlorite containing 2% 'Tween 20', for 20 minutes, washed in 3 changes of distilled water and placed in a dry sterile petri dish. Shoot tips (0.5-1mm long), consisting of an apical dome and one to two leaf primodia were excised under a dissecting microscope and used as explants.

2.3 Culture of Shoot-tip Explants

Single excised shoot tip explants were cultured in a universal bottle containing 10ml of medium. The medium consisted of MS (Murashige and Skoog, 1962) salts, B5 (Gamborg *et al*. 1968) vitamins, 30g/L sucrose, 8% agar and supplemented with various plant growth regulators as outlined below:

- TDZ (0.1, 0.4, 1.0 and 5.0 µM)
- 1.0 µM TDZ + NAA (0.01 and 0.1 mg/l)
- 1.0 mg/L BAP + 0.01 mg/L NAA
- 1.0 mg/L BAP + 0.1 mg/l NAA
- 0.1 and 1.0 mg/l BAP
- 0.01 and 0.1 mg/l NAA
- Basal medium devoid of any plant growth regulators acted as a control in all experiments (MSO).

Based on preliminary studies and those of Lin *et al.* (1997), explants were placed in culture media supplemented with TDZ, for a duration of 10 days, and subsequently
transferred to a basal medium devoid of any plant growth regulators. For all other treatments lacking any TDZ, explants were held continuously on the culture media.

Sub-culturing was done every 4 weeks. After 16 weeks in culture, the shoots were transferred to a rooting medium consisting of 3 mg/l IBA. The pH of the media was adjusted to 5.7± 0.1 before autoclaving at 121^0C and pressures of 1.19 kg cm^{-2} for 20 minutes. The cultures were placed on growth shelves set at 25 ± 2^0 C and illuminated (16 h photoperiod 70-78 µmol/m^2/s) by cool white fluorescent tubes. The number of shoots, shoot length and numbers of leaves/shoot were assessed every week for a period of 4 months (16 weeks).

2.4 Experimental Design and Statistical Analysis

All experiments were laid out in a completely randomized design and each treatment was replicated 3 times. All experiments were repeated twice and only 1 is reported as data were similar. Data were analyzed using the analysis of variance (GENSTAT) statistical package (Lane and Payne, 1996) and the means were compared using Tukey procedure at 5% level of significance.

3.0 RESULTS

3.1 Number of Shoots

The addition of various plant growth regulators to the induction media significantly influenced the number of shoots formed from shoot tip cultures of *Alstroemeria aurantica* cv. 'Rosita', over the 16 weeks of culture (Figure 1). A single shoot was formed on explants cultured on basal medium devoid of any plant growth regulators (MSO). Explants cultured in a media supplemented with a combined cytokinin (BAP) and auxin (NAA), at 1.0 and 0.01 mg/l, respectively, produced the largest number of shoots only comparable after 16 weeks by those cultured in media supplemented with 0.4 µM TDZ. Increasing the NAA concentration from 0.01 to 0.1 mg/L in the 1.0 mg/L BAP combination depressed shoot formation, as it did when combined with 1.0 µM TDZ. Shoot tip explants cultured in MS medium supplemented with NAA alone (0.01, 0.1 mg/l) or BAP alone (0.1, 1.0 mg/l) became necrotic and died after 7 days in culture (data not shown). Inclusion of various concentrations of TDZ in the culture medium increased the number of shoots formed per explant after 8 weeks in culture, the highest number (7) formed from shoot tips cultured on 0.4 µM TDZ after 16 weeks of culture. Unexpectedly, a single shoot was formed from explants cultured on media supplemented with 5.0 µM TDZ just as in basal medium devoid of any plant growth regulator.

3.2 Shoot Length

The inclusion of various plant growth regulators in the culture medium significantly increased the length of shoots formed *in vitro* from shoot tip explants of *Alstroemeria aurantiaca* cv. 'Rosita' (Figure 2). The longest shoots for the entire period of observation were those cultured on media supplemented with 1.0 µM TDZ followed by those on 5.0 µM TDZ. Incorporation of combined 1 mg/L BAP with different concentrations of NAA in the culture media also increased the shoot length. Increasing the NAA concentration to 0.1 mg/l in combination with the 1.0 mg/L BAP significantly reduced the shoot length. Low concentrations of TDZ on its own, or in combination with NAA caused a slight improvement in the shoot length after 4 weeks in culture. Shoots from explants maintained on basal medium devoid of any plant growth regulators were the shortest (< 0.5 cm) compared to the tallest measuring over 3.5 cm tall.

3.3 Number of Leaves

The addition of different concentrations of various plant growth regulators to the culture medium increased the number of leaves formed per explant (Figure 3). The addition of various PGRs increased the number of leaves per shoot. Explants cultured on media supplemented with 1.0 µM TDZ during the 16 weeks in culture, had the highest number of leaves (8) compared to those on basal medium, which had an average of only 1 leaf. Increasing TDZ concentration from 0.1 to 5.0 µM significantly improved leaf formation but there was no significant difference between 0.4 and 5.0 µM concentrations. Incorporation of combined BAP and NAA increased the number of leaves per shoot and though lower than those cultured on 1 µM TDZ were comparable to those raised on 5 µM TDZ. Addition of NAA to the 1 µM TDZ significantly decreased the number of leaves formed on each shoot of *Alstroemeria aurantiaca* shoot tip explants.

4.0 DISCUSSION AND CONCLUSION

Alstroemeria (A. aurantiaca L.) is a monocotyledonous crop whose *in vitro* propagation has proven difficult for many years due to what was originally believed to be its recalcitrant nature. In the current study, *Alstroemeria* was successfully maintained on basal medium devoid of any plant growth regulators. However, only a single short shoot was formed with very few leaves. The addition of various plant growth regulators significantly increased the number and length of shoots as well as the number of leaves per explant.

Thidiazuron is a phenyl urea that has gained importance as being more or just as potent as combined auxin and cytokinin in evoking morphogenic responses *in vitro* (Huetterman and Preece, 1993; Mok *et al*., 1982; Visser *et al*., 1992; Malik and Saxena, 1992; Murthy *et al*., 1995). In the present system, however, the best responses in terms of number of shoots, shoot length and number of leaves, was observed in explants cultured on basal medium supplemented with 1.0 mg/L BAP combined with low concentrations (0.01 mg/L) of NAA. Thidiazuron at concentrations of 0.4-1.0 µM was just as effective as combined auxin (NAA) and cytokinin (BAP) in influencing the number and length of shoots formed. The ability of TDZ and combined NAA and BAP to induce multiple shoot formation and increased shoot length has been documented in other systems (Kerns and Meyer, 1986; Fellman *et al*., 1987; Fiola *et al*. 1990). Naphthalene acetic acid and BAP alone were ineffective in evoking significant morphogenic responses from shoot tip explants of *Alstroemeria*. Optimum responses were achieved when BAP was combined with NAA at low concentrations (0.01 mg/L) in the culture media. However, higher auxin levels significantly reduced the number of shoots formed but not the shoot length or number of leaves formed per shoot. Inclusion of NAA in the TDZ-supplemented culture medium reduced the shoot length and number of leaves formed but had no effect on the actual number of shoots formed. The results of this study are in contrast to reports of 10.0 µM TDZ + 0.5 µM IBA enhancing shoot regeneration from leaf explants of *Alstroemeria* (Lin *et al*. 1997), although the numbers reported are similar to those achieved at 0.4 µM TDZ alone in this study after 14 weeks of culture.

Unexpectedly though, a high concentration of TDZ (5.0 µM), although supporting shoot growth, did not promote multiple shoot formation. Thidiazuron has been reported to modulate endogenous levels of plant growth hormones (Murthy *et al*. 1995; Hutchinson and Saxena, 1996; Hutchinson *et al*. 1996a; b), including auxin, ethylene and cytokinins. Probably at high concentrations, TDZ could have influenced phytohormone concentrations and/or ratios especially of auxins and cytokinins to levels that promote apical dominance (Bond and Alderson, 1993) at the expense of lateral shoot proliferation. Supra-optimal levels of PGRs have been shown to inhibit morphogenic responses, possibly through negative feed-back mechanism. Thidiazuron could have inhibited growth through elevation of endogenous ethylene, a hormone that has been reported to promote degradative processes, in addition to causing stem shortening and thickening (Beyer *et al*. 1984; Eisinger, 1983). Naphthalene acetic acid and BAP alone were ineffective in evoking significant morphogenic responses from shoot tip explants of *Alstroemeria aurantiaca* cv. 'Rosita'.

The results of this study suggest that morphogenic responses in plants are regulated by an intricate balance and interaction of various phytohormones, namely auxins, cytokinins, ethylene, gibberellic acid and possibly ABA (Trewavas, 1981; Hutchinson, 1996). Thidiazuron could have modulated elevated levels of auxins, cytokinins and ethylene as reported in other plant systems e.g. geranium (Hutchinson *et al*., 1996b), peanut (Murthy *et al*., 1995). High auxin and ethylene levels inhibit shoot elongation in several systems (Suttle, 1985; Beyer *et al*., 1984). An aspect of

competition for space or nutrients by the regenerated shoots (Hartmann *et al*., 1990) cannot be ruled out in the present cultures.

In conclusion, TDZ at low concentrations was as effective as combined auxin (NAA) and cytokinin (BAP) in evoking shoot regeneration and elongation as well as the number of leaves formed per shoot during *in vitro* propagation of *Alstroemeria aurantiaca* cv. 'Rosita' from shoot tip explants.

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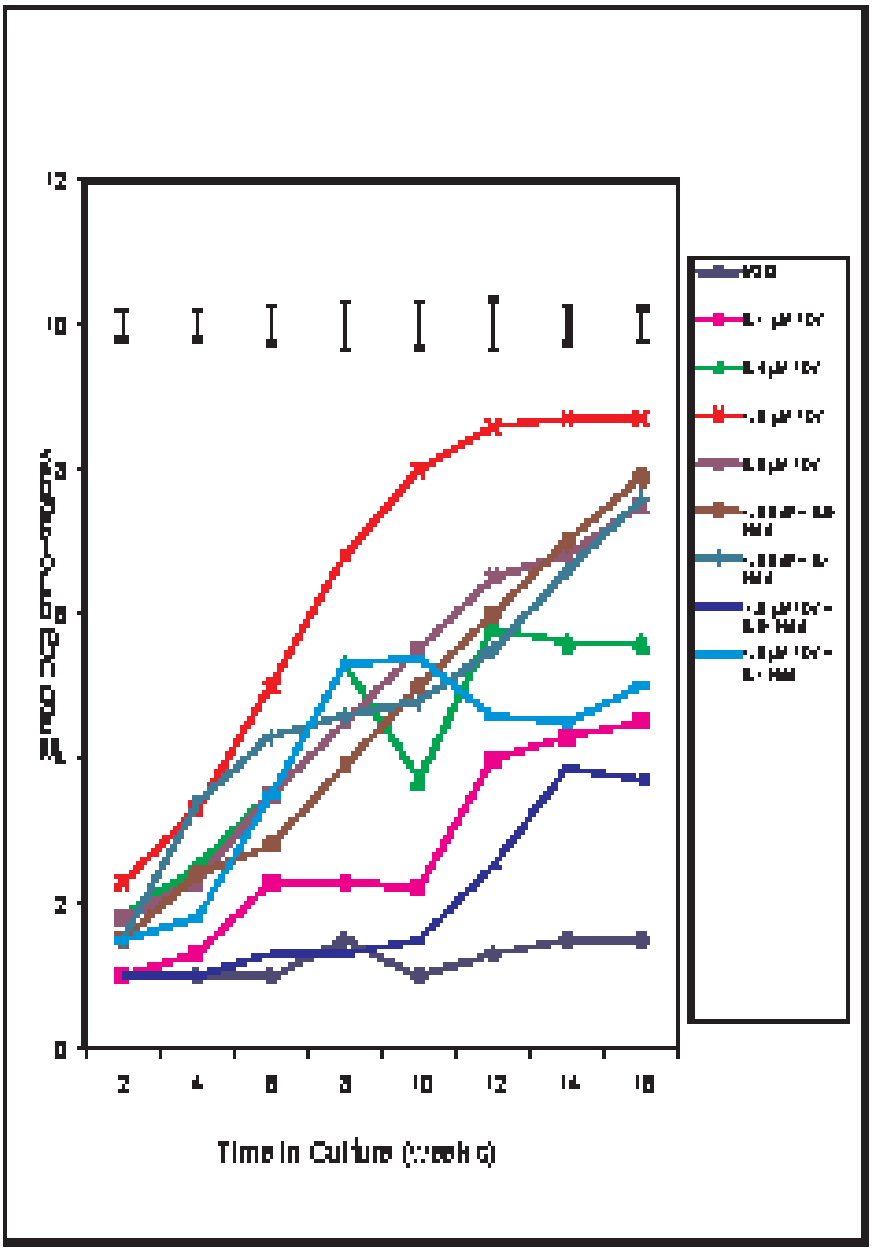

Figure 1: Effect of TDZ, NAA and BAP on number of leaves per shoot on [illegible] cultures in Murashige and Skoog's basal medium

Key: Mean separation using Tukey's method and vertical bars represent standard errors at 5% level of significance