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Effects of Cytokinin Type and Concentration on Shoot Proliferation in a Novel *Tripidium* Hybrid

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Summary

Tripidium, a genus within the sugarcane complex (Andropogoneae tribe of the Poaceae), is used as a landscape plant and more recently has been recognized for its bioenergy potential. Micropropagation protocols were investigated to expedite shoot proliferation. Four cytokinin types including 6-benzylamino purine (BAP), thidiazuron (TDZ), zeatin and kinetin were investigated at concentrations of 0. 2.5, 5.0, 10.0 or 20.0 µM. In a second experiment the effect of BAP or TDZ, alone or a 1:1 ratio combination, at 2.5, 5.0, or 10.0 µM on shoot regeneration was investigated. Media supplemented with either 5 µM TDZ or 20 µM BAP produced 6.05 and 5.75 shoots per explant, respectively. The combination of BAP and TDZ did not significantly improve multiplication rates. This research provides protocols for rapid multiplication and micropropagation of *Triipidium.*

INTRODUCTION

Tripidium is a genus within the Poaceae Subtribe Saccharinae, commonly referred to as the sugarcane complex. New interspecific hybrids between *Tripidium ravennae* and *T. arundinaceum* have considerable potential as biomass crops and have demonstrated high yields and overwintering survival rates in USDA Zone 6b (Maren et al., 2021). Traditionally these bioenergy grasses have been commercially propagated through divisions or rhizomes. However, newly developed high-yielding varieties show dense clumping rhizomatous masses with increased culm densities. While these qualities have led to increased yields, they have also led to reduced efficiency of conventional propagation and field establishment methods using divisions and rhizomes. Development of in vitro propagation methods would provide a valuable option for more rapid multiplication.

While micropropation studies on *Tripidium* have been limited (da Silva et al., 2020), there have been several reports on related genera within the sugarcane complex including *Saccharum* sp. (Jahangar et al. 2010; Ramgareed et al., 2010; Salokhe, 2021) and *Miscanthus* sp. (Zhang et al., 2010). In these studies, 6-benzylamino purine (BAP) alone has been the most predominant cytokinin for effective shoot proliferation, though optimal concentrations varied among genera. For *Saccharum* sp., concentrations between 1 to 5 μM BAP were effective for shoot proliferation (Ajadi et al., 2018; Jahangir et al., 2010), while for *Miscanthus* 10-20 μM BAP were more effective (Zhang et al., 2012). However, for some *Saccharum* sp. low concentrations of kinetin in combination with BAP have been utilized to improve shoot proliferation (Balagalla et al., 2018; Cheong et al., 2009; da Silva et al., 2020).

Zeatin and TDZ have been effectively used to a lesser extent to induce shoot proliferation in species within the sugarcane complex. For example, Vinayak et al. (2009) found zeatin to be the most effective cytokinin for shoot proliferation for *Saccharum spontaneum* hybrids. Vazques-Molina et al. (2005) found TDZ to be effective for shoot proliferation in sugarcane (*Saccharum* spp,) cultivars. Interestingly, Sukendah et al. (2023) found that a combination of TDZ and BAP was most effective for shoot proliferation in several *Saccharum* sp. genotypes.

With the development of *Tripidium* as a bioenergy feedstock it would be beneficial to develop micropropagation protocols to facilitate rapid propagation. Considering the variation in media used for related species, the objective of this research was to investigate the effect of cytokinins type and concentration on shoot proliferation of a novel *Tripidium* hybrid.

MATERIAL AND METHODS

Plant material and culture conditions. Nodal sections with actively growing axillary shoots were used to initiate in vitro cultures. Actively growing shoots were collected from field grown plants and rinsed under tap water for 4 h. Explants were surface-disinfested in 20% (v/v) bleach (6.15% NaOCl) solution containing two to three drops of Tween® 20 for 25 min with periodic mixing followed by three 5-min rinses in sterile distilled water. Explants were cultured on initiation medium consisting of Murashige and Skoog (MS) basal salts and

vitamins (Murashige and Skoog, 1962) supplemented with 20 µM BAP, 100 mg/L *myo*-Inositol, 100 mg/L 2-(N-Morpholino) ethanesulfonic acid (MES) monohydrate, and 30 g/L sucrose. Media were solidified with 6.5 g/L agar (Phytotechnology Laboratories, Shawnee Mission, KS) and adjusted to pH of 5.75, and 25 mL was dispensed to 180-mL glass jars. Microshoots were maintained by transfer to fresh culture medium every 4 to 6 weeks and incubated under standard culture conditions [23° ± 2°C and a 16 h photoperiod of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps].

Cytokinin Type and Concentration. Effects of cytokinins and their concentration on microshoot growth and proliferation were examined. Media consisted of MS salts and vitamins supplemented with BA, TDZ, zeatin, or kinetin at 0, 2.5, 5.0, 10.0 or 20.0 µM. Cytokinins were added to media prior to autoclaving, except for zeatin, which was filter sterilized and added to cool autoclaved media. All media were supplemented with 100 mg/L *myo*-Inositol, 100 mg/L MES, and 30 g/L sucrose, solidified with 6.5 g/L agar and adjusted to pH 5.75. Media (25 mL) was dispensed into 180-mL glass jars. Three-week- old actively growing stock plants were divided into single shoots and placed onto experimental media. Five microcuttings (10–20 mm long) were placed vertically in each jar. Eight replicates of each media composition were incubated under standard culture conditions, as described previously, in a completely randomized design. After 8 weeks, data were recorded on the number of surviving explants, number of microshoots, and microshoot length (of longest shoot). Data sets were subjected to regression analysis.

BAP and TDZ Combinations. The effects of the cytokinin BAP and TDZ, alone or in combination, on shoot production was investigated. Basal media for all treatments was MS salts and vitamins, 6.5 g/L of agar, 0.1 g/L of Myo-inositol, 0.1 g/L of MES buffer, and 30 g/L of sucrose. To test the effects of cytokinins, media were supplemented with either BAP, TDZ, or a 1:1 ratio of each at 0, 2.5, 5, or 10 µM. Media was dispensed into 180 jars with approximately 25 ml in each jar. Three-week-old actively growing stock plants were divided into single shoots and placed onto experimental media. Each treatment consisted of 8 reps (jars) each containing 5 subsamples (shoots) and were cultured under standard culture conditions as previously described in a completely randomized design. After eight weeks data can be collected on explant survival shoot number and length. Data sets were subjected to regression analysis.

Rooting. Media used for in vitro rooting consisted of half strength MS salts and vitamins supplemented with 5 µM NAA, 30 g/L sucrose, 100 mg/L MES, and 100 mg/L *myo*-inositol. Media were solidified with 6.5 g/L agar and adjusted to a pH of 5.75. Microcuttings, 10 to 20 mm long, were subcultured on 25 mL of media in 180-mL jars. All jars were incubated under standard culture conditions as described previously. Following 2 weeks of growth, microshoots were transferred ex vitro.

RESULTS AND DISCUSSION

Cytokinin Type and Concentration. Shoot multiplication was achieved for all treatments. There were significant interactions between cytokinins and their concentration that affected explant survival ($P < 0.05$) and shoot production ($P < 0.05$). Explant survival was reduced on basal media and increased in the presence of cytokinins. For BAP and kinetin, explant survival also

declined at higher concentrations (**Fig. 1a**). In general, shoot production was highest on media containing BAP or TDZ (**Fig. 1b**). For BAP, shoot production followed a quadratic model with the highest number of shoots per explant reaching 5.73 ± 0.75 at 20 µM. Similarly, for TDZ shoot proliferation followed a quadratic model, reaching maximum shoot proliferation at 10 µM and then declines with increasing concentration. There was no effect of cytokinin or their concentration on shoot length.

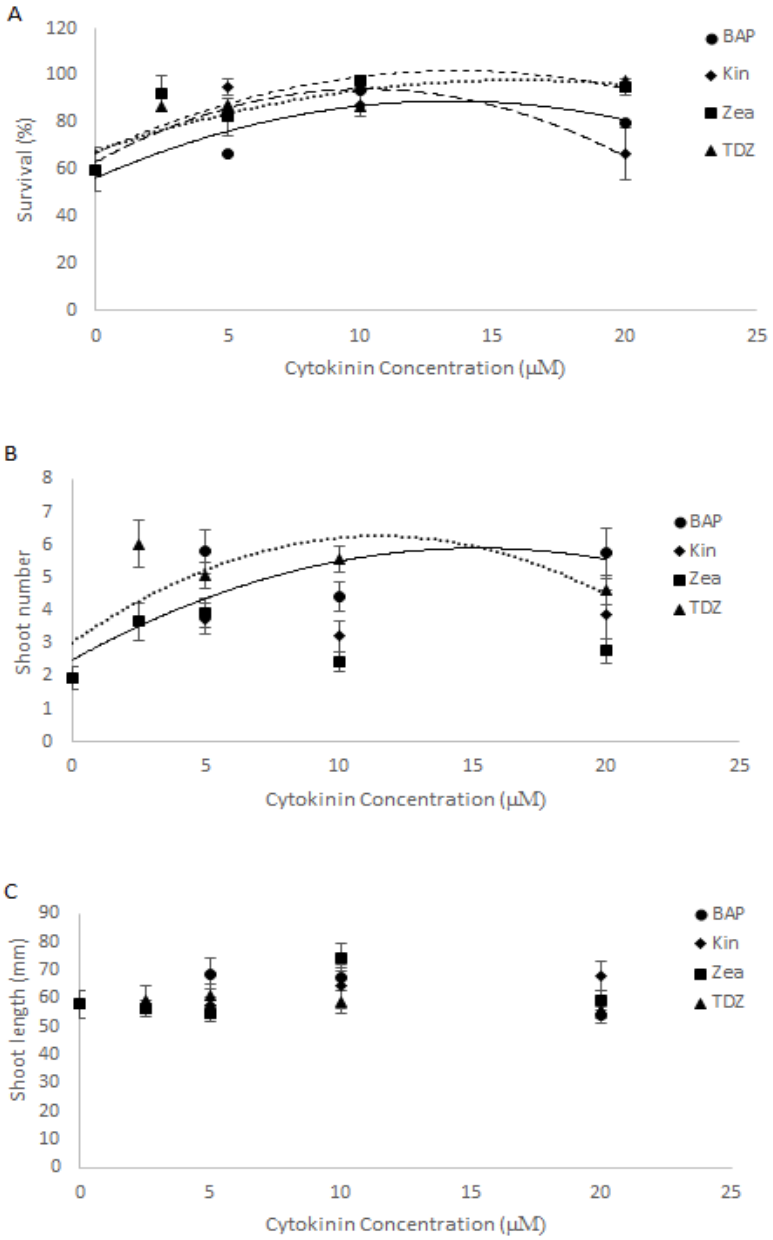

Figure 1. A) Influence of cytokinin type and concentration on explant survival. Symbols represent means, (n=8, ± SEM). BAP, $y = 0.57 + 0.05x - 0.002x^2$; Kinetin, $y = 0.63 + 0.06x - 0.003x^2$; Zeatin, $y = 0.67 + 0.05x - 0.002x^2$; TDZ, $y = 0.66 + 0.04x - 0.001x^2$. B) Shoot number as a function of cytokinin type and concentration. Symbols represent means, (n=8, ± SEM). BAP, $y = 2.38 + 0.44x - 0.01x^2$; Kinetin, NS; Zeatin, NS; TDZ, $y = 2.18 + 0.6x - 0.03x^2$. C) Shoot length as a function of cytokinin type and concentration. Symbols represent means, (n=8, ± SEM).

Interestingly, both zeatin and kinetin resulted in relatively low shoot proliferation rates. Kinetin, in particular, has been widely used for genera within the sugarcane complex. For *Saccharum officinarum*, maximum shoot regeneration was obtained using BAP in combination with kinetin (Salokhe, 2021; Shimelis et al., 2014; Tesfa et al., 2016).

BAP and TDZ Combinations. Shoot multiplication was achieved for all treatments. There were significant interactions between cytokinins and their concentration that affected explant survival ($P < 0.05$) and shoot production ($P < 0.05$), but not shoot length. Explant survival declined in a quadratic model when exposed to increasing concentrations of BAP and TDZ (**Fig. 2a**).

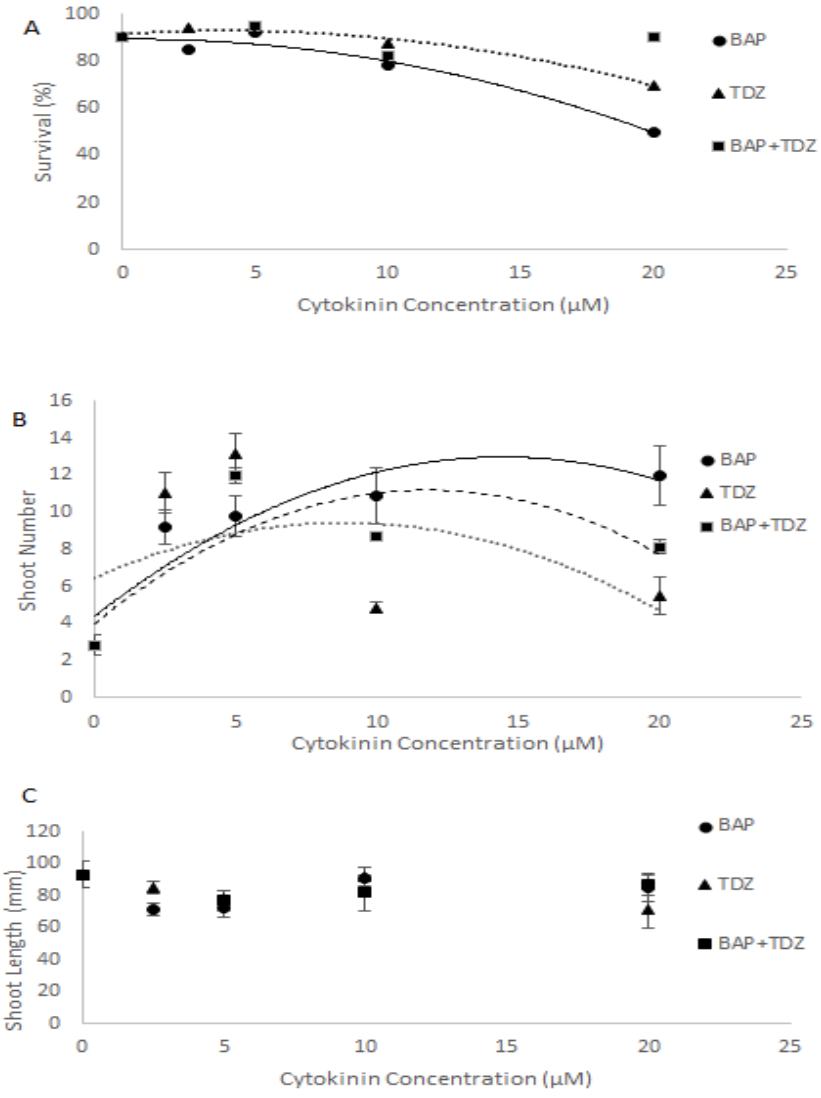

Figure 2. A) Influence of cytokinin type and concentration on explant survival. Symbols represent means, (n=8, ± SEM). BAP, $y = 88.3 + 0.95x - 0.09x^2$; TDZ, $y = 89.3 + 0.105x - 0.104x^2$; BAP+TDZ, NS. B) Shoot number as a function of cytokinin type and concentration. Symbols represent means, (n=8, ± SEM). BAP, $y = 4.4 + 1.2x - 0.04x^2$; TDZ, $y = 6.48 + 0.67x - 0.038x^2$; BAP+TDZ, $y = 4.0 + 1.2x - 0.05x^2$. C) Shoot length as a function of cytokinin type and concentration. Symbols represent means, (n=8, ± SEM).

Explant survival remained high across all concentrations in response to a combination of BAP and TDZ (**Fig. 2a**). High concentrations of cytokinins are known for inducing programmed cell death and could be attributed to the reduction in survival. However, it is interesting that the same response what not observed when using a combination of cytokinins.

Regression analysis showed shoot production followed quadratic responses to concentration. For both TDZ and the combination of BAP and TDZ, shoot production increased until 10 µM before attenuating with increased concentrations. For BAP, shoot production followed a quadratic model and produced the highest number of shoots per explant reaching 11.98 ± 1.6 at 20 µM (**Fig. 2b**). The use of BAP as the sole cytokinin has been successful for shoot proliferation in numerous studies on plants in the sugarcane complex. However, in a more recent study, Sukendah et al. (2023) explored different ratios of BAP and TDZ and found a combination of 15 µM BAP and 2 µM TDZ produced the highest number of shoots. In contrast, in the present study, combinations of BAP and TDZ were not as effective as BAP alone. However, this study used concentrations of BAP and TDZ in a 1:1 ratio (i.e., 2.5 µM BAP: 2.5 µM TDZ). Exploring different ratios of BAP and TDZ may be more beneficial for increasing proliferation rates.

Microcuttings initiated roots within 7 to 10 days after being placed into rooting media. After 2 to 4 weeks plantlets had significant root formation (**Fig. 3**) and were transferred to greenhouse conditions under mist.

Figure 3. In vitro root formation on *Tripidium* before transferring ex vitro.

CONCLUSIONS

Cytokinins play an important role for in vitro shoot proliferation. Considering the variability in cytokinins used for species and cultivars within the sugarcane complex, this study describes the refinement of cytokinins used for shoot proliferation in a novel *Tripidium* hybrid. Both BAP and TDZ were shown to be effective cytokinins for shoot proliferation. These protocols provide a basis for rapid propagation of *Tripidium* and further enhance the genera as a potential bioenergy feedstock.

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