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# Plantlet regeneration from young leaf segments of curaua (*Ananas erectifolius*), an Amazon species

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**Abstract:** The present study reports the first efficient in vitro regeneration of *Ananas erectifolius* via indirect organogenesis. Leaf segments (leaf base, middle, and apex) excised from 3- or 5-week-old in vitro plantlets were cultured on 1/4 strength MS medium supplemented with different concentrations and combinations of plant growth regulators. The explants were also exposed to pulse treatment with thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) for different periods of time. The results showed that using juvenile rather than old explants enhanced the frequency of callus induction (35.0% and 16.0%, respectively). Among the explant types tested, only leaf base segments induced calli; the highest frequency occurred via culture treatment containing 4-amino-3,5,6-trichloropicolinic acid (picloram; 48.57%), 2,4-D (40%), or TDZ (35.71%). However, only calli generated in treatments containing TDZ or $N^6$-isopentenyladenine (2ip) were able to develop shoots (maximum 35.71% and 14.28%, respectively). A combination of TDZ and 2,4-D improved callus induction (60.0%) but did not increase shooting response. However, both callusing and shooting response increased when 10 days of pulse treatment with TDZ and 2,4-D was applied (66.83% and 48.7%, respectively). Rooted plantlets that exhibited normal growth and development were acclimatized in a greenhouse and had a survival rate of 95%.

**Key words:** *Ananas erectifolius*, callus induction, in vitro regeneration, pulse treatment, thidiazuron

## 1. Introduction

*Ananas erectifolius* (L.B.Sm.), a member of the family Bromeliaceae, is native to the Amazon region where it is known as curaua. This species is very closely related to pineapple (*Ananas comosus*), can grow within a range of 1 to 1.5 m, and produces at least 12 to 15 leaves (Pereira et al., 2008). Apart from being an edible and medicinal plant, it has been used for a range of purposes. Fiber produced from *A. erectifolius* leaves is one of the most resilient plant fibers (González-Olmedo et al., 2005). In Brazil, the market demand for natural fibers is very high and it provides job opportunities for more than one million citizens, particularly those living in undeveloped areas (Lameira et al., 2003).

Due to the economic importance of resilient fiber used in the textile and automobile industries and by civil construction companies, many studies have focused on improving the quality and production of *A. erectifolius*. Studies by Pereira et al. (2008) indicated that, by virtue of its resistance, softness, and lightness, *A. erectifolius* fiber is particularly suitable for the internal trimming of cars (i.e. seat covers and carpets). Nevertheless, the industrial application of *A. erectifolius* fiber is limited due to an insufficient supply of raw materials. Because *A. erectifolius* can only grow in humid and warm climates, acquisition of propagative material outside of such regions is difficult (Teng, 1997; González-Olmedo et al., 2005; Pereira et al., 2008). *Ananas erectifolius* is generally propagated by conventional agricultural methods through scions from the mother plant in the field. However, this method has increased disease susceptibility with successive plantings and leads to low productivity (Lameira et al., 2003). In order to overcome these limitations, cell or tissue culture methods can be a good alternative for producing large quantities of disease-free propagules (Lameira et al., 2003; Sripaoraya et al., 2003; Pereira et al., 2008).

In vitro propagation of different plant species from leaf segments has been described (e.g., Kackar et al., 1993; Sripaoraya et al., 2003; Gill et al., 2006; Pola and Mani, 2006; Li et al., 2010; Chen et al., 2014; He and Gang, 2014; Kaur and Sandhu, 2015; Moon et al., 2015; Raju et al., 2015). The only report addressing micropropagation of

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*A. erectifolius* was a study by Pereira et al. (2008), which reported the effects of etiolation and growth regulators on bud elongation and plantlet regeneration. However, no study regarding in vitro propagation of *A. erectifolius* from leaf segments has been reported. Due to the commercial potential of *A. erectifolius* and the necessity of improving our understanding of in vitro propagation in this important species, the current work presents an effective method for in vitro regeneration of *A. erectifolius* using young leaf segments. The effects of different types and concentrations of plant growth regulators (PGRs) and the factors influencing callus induction and multiple shoot regeneration are discussed as well.

## 2. Materials and methods

### 2.1. Explant source

In vitro samples of *A. erectifolius* were collected from the Botanical Garden of Embrapa Amazonia Oriental (Belem, Para, Brazil), and voucher specimens were preserved in the herbarium under reference number N. 1 (IAN 178667). The plantlets were regenerated from axillary buds cultured on semisolid MS medium (Murashige and Skoog, 1962) without PGRs and incubated at 26 ± 1 °C under a 16/8-h light/dark photoperiod (25–30 µmol photons $m^{-2}$ $s^{-1}$) supplied by cold fluorescent bulbs.

To determine the best explant for callus induction, leaves obtained from 3- and 5-week-old in vitro plantlets (young and old leaves) were used as explants. The leaves were cut into three different segments: leaf base, middle, and apex (Figure 1). The leaf segments (0.5 × 1.0 cm) were aseptically inoculated in jars (250 mL) containing 40 mL of 1/4 strength MS medium supplemented with 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D). The medium was prepared with 3% (w/v) sucrose, solidified with 0.6% (w/v) agar, and adjusted to pH 5.7 with 0.5 M HCl or 0.5 M NaOH prior to autoclaving at 120 °C for 15 min. The cultures were maintained for 30 days in a growth room at 26 ± 1 °C under a 16/8-h light/dark photoperiod provided by cool-white fluorescent light (Osram, Brazil) with an irradiance of 25 µmol photons $m^{-2}$ $s^{-1}$.

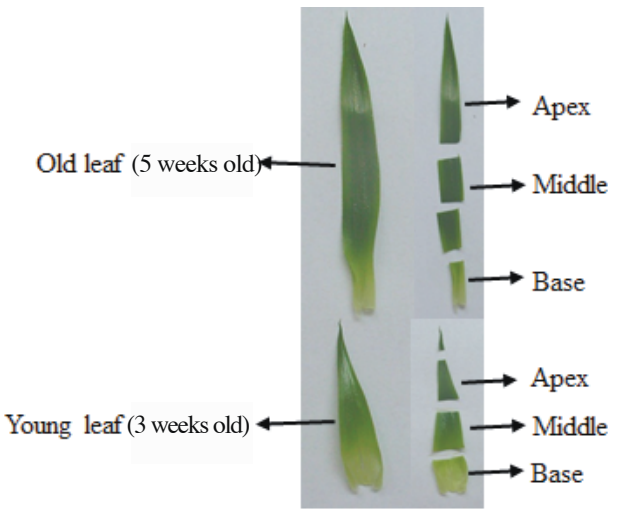


**Figure 1.** Leaf explants excised from different parts (apex, middle, and base) of young and old leaves of *Ananas erectifolius*.

### 2.2. Callus induction and plantlet regeneration

For testing the effects of different concentrations of growth regulators on callus induction and shoot regeneration, only leaf base segments (explants) excised from 3-week-old in vitro plantlets were used throughout the experiment. The explants (0.5 × 1.0 cm) were cultured on 1/4 strength MS medium containing 0, 2.5, 5.0, 10.0, or 20.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (picloram), thidiazuron (TDZ), $N^6$-benzylaminopurine (BAP), or $N^6$-isopentenyladenine (2ip). In addition to using an individual growth regulator at various concentrations, another set of explants were incubated in 1/4 strength MS medium containing different concentrations (0, 2.5, 5.0, 10.0, or 20.0 µM) of BAP or TDZ, each supplemented with 20.0 µM 2,4-D. The media were prepared with 3% (w/v) sucrose and 0.6% (w/v) agar and autoclaved at 120 °C for 15 min after adjusting the pH to 5.7. The cultures were maintained at 26 ± 1 °C under a 16/8-h light/dark photoperiod supplied by cool-white fluorescent light with an irradiance of 25 µmol photons $m^{-2}$ $s^{-1}$. In all treatments, the callus-containing shoot clusters and original explants were maintained with their respective treatments for 6 weeks before transferring the shoots to rooting media.

### 2.3. Pulse treatment with TDZ and 2,4-D

Short-time contact of explants with growth regulator (pulse treatment) was established using leaf base segments (0.5 × 1.0 cm) obtained from 3-week-old plantlets. The explants were pulse-treated on 1/4 strength MS medium containing 10 µM TDZ and 20 µM 2,4-D for 5, 10, 15, 20, or 25 days. After each pulse treatment, the explants were transferred to 1/4 strength MS medium without growth regulator and maintained at room temperature under a 16/8-h light/dark photoperiod provided by cool-white fluorescent light with an irradiance of 25 µmol $m^{-2}$ $s^{-1}$. After 30 days, the percentages of callus induction and shoot regeneration were recorded before transferring the shoots to rooting media.

### 2.4. Histological studies

Shoot regeneration from the callus clumps was confirmed using scanning electron microscopy (SEM; LEO Evo 40 XVP, Cambridge, UK). The calli with induced shoot buds were fixed in Karnovisk solution (pH 7.2) for 24 h. The samples were washed with cacodylate buffer (pH 7.2) three times and fixed in 1% (w/v) osmium tetroxide at room

temperature for 4 h. After that, the samples were washed with distilled water and dehydrated through a graded acetone series [25, 50, 75, 90, and 100% (v/v) acetone] 3 times for each concentration. Finally, the dehydrated samples were dried and coated with gold–palladium using a Quick Cool Coater (Sanyu-Denshi, Japan), and then they were examined by SEM.

### 2.5. Rooting and acclimatization

After successful shoot regeneration from the callus, the shoots were separated individually and cultured on 1/4 strength MS medium without plant growth regulator for rooting. The rooting experiment was maintained at room temperature under a 16-h photoperiod supplied by cool-white fluorescent light with an irradiance of 25 µmol photons $m^{-2}$ $s^{-1}$. The shoots were maintained in the medium for 30 days in order to produce sufficient roots and for further development into mature plantlets. Afterwards, the plantlets (approximately 6 cm with 4–5 leaves) with well-developed roots were gently removed from the rooting medium. Roots were washed under running tap water to clean the agar. After that, the plantlets were transferred to plastic pots (20 × 25 cm) filled with commercial compost (Plantmax, São Paulo, Brazil) containing a mixture of vermiculite, organic matter, and sand in a ratio of 1:2:1, and were acclimatized in a greenhouse at ambient temperature under 50% shading and 60% relative humidity.

### 2.6. Data collection and analysis

The frequency of callus induction was presented as the percentage of calli out of the total number of noncontaminated explants of three independent repeats. The frequency of shoot regeneration was presented as the percentage of shoots from the total number of calli clumps of the three independent repeats. The experiments were repeated three times with 12 replicates in each. All data were statistically analyzed using SISVAR statistical software (Ferreira, 2007), and the mean frequency difference was compared using the Scott–Knott test (at 95% confidence interval). Variability in frequency was presented as ±standard error.

## 3. Results and discussion

### 3.1. Effect of explant source

Selection of the best explants for callus induction and shoot regeneration has been the most critical factor in the micropropagation of many plant species (Sripaoraya et al., 2003; Mohammed et al., 2015; Takamori et al., 2015; Yücesan et al., 2015). Therefore, to select the best explant for indirect organogenesis in *A. erectifolius*, different types of explants were tested. The results revealed a significant difference in terms of frequency of callus induction between the explant ages (3- and 5-week-old leaves) as well as the positions from which explants were excised (leaf base, middle, and apex). Calli were induced only on base segments, with a maximum of 35% frequency in callus induction among young leaves and 16% among old leaves (Table 1). There was no callus induction or shoot regeneration from the leaf apex or middle segments in young or old leaves.

Different responses among explants were probably due to the the endogenous hormone balance in the plant tissues. Because leaf base segments have younger and less differentiated cells with higher meristematic activities than the leaf apex and middle segments, the explants obtained from base segments showed high callusing response (Asghari et al., 2012). Another important factor is that cells in base segments may have a great number of receptors for the growth regulator present in the culture medium (Close and Gallagher-Ludeman, 1989), which may be the factor influencing callus induction in *A. erectifolius*. Galiba et al. (1986) proposed that a polygenic system might also be involved in the determination of organogenesis. Similar to our results, many studies have reported the effectiveness of young leaves in callus induction (Yang et al., 2014; Kaur and Sandhu, 2015; Moon et al., 2015; Raju et al., 2015).

### 3.2. Effect of growth regulators on callus induction and shoot regeneration

After identifying the best explant for callus induction (Figure 2A), the effects of different concentrations (2.5–20 µM) of various plant growth regulators on callus induction

**Table 1.** Frequency (%) of callus induction from different explants of *Ananas erectifolius* after 30 days of culture.

| Types of explant (leaf) | Frequency of callus induction on different leaf segments (%) | | |
|---|---|---|---|
| | Base | Middle | Apex |
| Young (3 weeks old) | 35.00 ± 1.5[a] | 0.00 ± 0.0[a] | 0.00 ± 0.0[a] |
| Old (5 weeks old) | 16.00 ± 1.2[b] | 0.00 ± 0.0[a] | 0.00 ± 0.0[a] |

Frequencies followed by the same lowercase letter in a column are not significantly different by the Scott–Knott test ($P \leq 0.05$). The data represent the mean frequency ± standard error (SE) of 3 independent repeats.

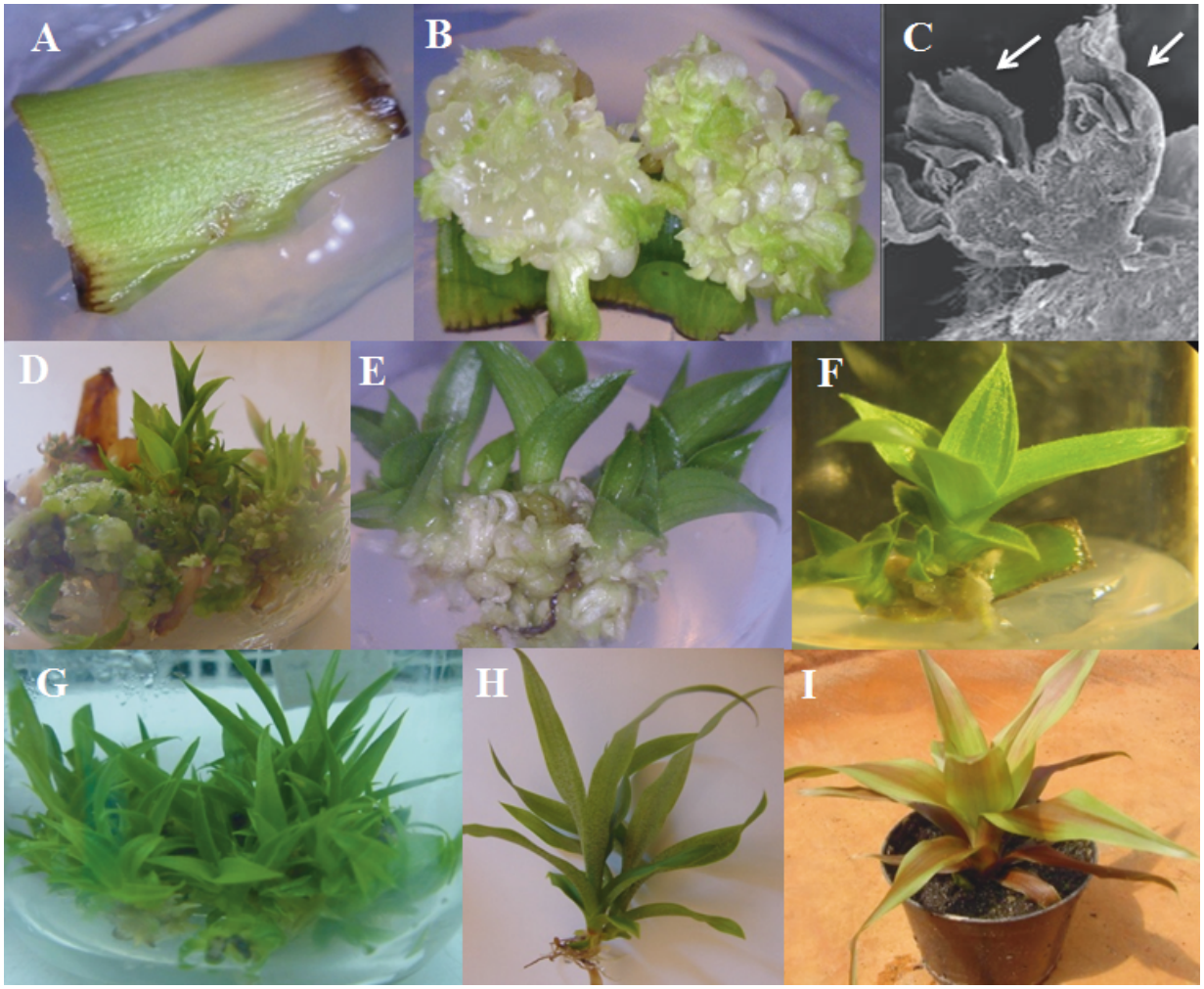


**Figure 2.** Micropropagation of *Ananas erectifolius* via indirect organogenesis from leaf base segment obtained from young leaves. A) Explant excised from leaf base; B) callus induced on treatment containing TDZ; C) initiation of shoot formation (see arrow) from callus, taken with scanning electron microscope; D. and E) further shoot growth on the same medium; F. and G) regenerated shoots on 1/4 strength MS medium; H) regenerated plantlet with well-developed roots; I) acclimatized plantlet in greenhouse conditions.

and shoot regeneration were tested. The results of this investigation revealed that medium formulation has a significant effect on frequency of callus induction and shoot regeneration. High frequencies of callus induction were found on medium containing picloram (48.57%), 2,4-D (40%), and TDZ (35.71%) (Table 2; Figure 2B). Treatments containing 2ip or BAP were less effective, resulting in only 17.14% and 5.71% callus induction, respectively. This suggests that picloram, 2,4-D, and TDZ were better suited for callus induction than 2ip or BAP in *A. erectifolius*. Similarly, Li et al. (2010) reported that TDZ induced a higher frequency of callus formation than BAP in *Hemerocallis lilioasphodelus* L.

After callus production, shoot formation was observed in treatments supplemented with TDZ or 2ip; however, treatments containing TDZ produced more shoots than 2ip. The highest frequency of shoot regeneration (maximum 35%) was obtained on MS medium containing 10 µM TDZ (Figures 2C–2E). Calli produced in picloram, BAP, or 2,4-D treatments did not induce shooting, indicating that these growth regulators were unable to produce shoots from the calli. Therefore, treatments containing picloram, 2,4-D, or BAP were only effective for callus induction and were ineffective for shoot regeneration in *A. erectifolius*. In the control treatment, no callus or shoot formation was observed (Table 2). The initial calli produced presented two different colors depending on the treatment; yellowish and mucilaginous calli were observed in treatments containing 2,4-D, BAP, and picloram, while white and compact calli were observed in the treatments containing TDZ and 2ip.

**Table 2.** Influence of different growth regulators at various concentrations on frequency of callus induction and shoot regeneration from leaf base segments of young leaves (3 weeks old) of *Ananas erectifolius*.

| Growth regulator | Concentration (µM) | Frequency of callus induction (%) | Frequency of shoot regeneration (%) |
|---|---|---|---|
| MS (control) | - | $0.00 \pm 0.0^{i}$ | $0.00 \pm 0.0^{h}$ |
| 2,4-D | 2.5 | $17.14 \pm 0.5^{f}$ | $0.00 \pm 0.0^{h}$ |
| | 5 | $31.43 \pm 1.2^{d}$ | $2.86 \pm 0.4^{g}$ |
| | 10 | $40.00 \pm 1.0^{b}$ | $0.00 \pm 0.0^{h}$ |
| | 20 | $35.00 \pm 0.5^{c}$ | $2.86 \pm 0.3^{g}$ |
| TDZ | 2.5 | $34.28 \pm 0.4^{c}$ | $31.71 \pm 1.0^{b}$ |
| | 5 | $24.28 \pm 0.6^{e}$ | $24.28 \pm 0.9^{c}$ |
| | 10 | $35.71 \pm 1.1^{c}$ | $35.71 \pm 1.2^{a}$ |
| | 20 | $24.28 \pm 0.8^{e}$ | $18.57 \pm 1.1^{d}$ |
| 2ip | 2.5 | $8.57 \pm 0.5^{gh}$ | $8.57 \pm 0.8^{f}$ |
| | 5 | $14.28 \pm 1.3^{fg}$ | $14.28 \pm 1.0^{e}$ |
| | 10 | $17.14 \pm 1.1^{f}$ | $11.43 \pm 0.9^{ef}$ |
| | 20 | $11.43 \pm 1.5^{g}$ | $8.57 \pm 0.4^{f}$ |
| BAP | 2.5 | $2.86 \pm 1.1^{hi}$ | $0.00 \pm 0.0^{h}$ |
| | 5 | $5.71 \pm 0.9^{h}$ | $0.00 \pm 0.0^{h}$ |
| | 10 | $2.86 \pm 0.6^{hi}$ | $2.86 \pm 0.4^{g}$ |
| | 20 | $5.57 \pm 1.3^{h}$ | $2.86 \pm 0.6^{g}$ |
| Picloram | 2.5 | $48.57 \pm 1.5^{a}$ | $0.00 \pm 0.0^{h}$ |
| | 5 | $40.00 \pm 1.3^{b}$ | $0.00 \pm 0.0^{h}$ |
| | 10 | $25.71 \pm 1.1^{de}$ | $0.00 \pm 0.0^{h}$ |
| | 20 | $27.14 \pm 0.7^{d}$ | $0.00 \pm 0.0^{h}$ |

Frequencies followed by the same lowercase letter in a column are not significantly different by the Scott–Knott test ($P \leq 0.05$). The data represent the mean frequency ± standard error (SE) of 3 independent repeats.

Shoot regeneration was noted only in white and compact calli (TDZ and 2ip treatments).

The success of TDZ in regenerating shoots from the calli of *A. erectifolius* might be because TDZ has both auxin and cytokinin activities, unlike natural cytokinins (Mohammed et al., 2015). This is in accordance with a report by Guo et al. (2011), which stated that TDZ has auxin- and cytokinin-like effects in different plant species. Picloram, BAP, and 2,4-D were ineffective for shoot regeneration from calli in *A. erectifolius*; however, many studies have reported successful application of these growth regulators for enhancing shoot formation in strawberry (Kordestani and Karami, 2008), *Urochloa* species (Takamori et al., 2015), *Vitis vinifera* (Dai et al., 2015), *Torenia fournieri* (Chanchula et al., 2015), *Pelargonium sidoides* (Kumar et al., 2015), and *Cordyline australis* (Warchoła et al., 2015).

Furthermore, the synergistic effects of different combinations of 2,4-D and BAP or TDZ on callus induction and shoot regeneration were investigated. The frequency of callus induction increased significantly using TDZ or BAP in combination with 2,4-D (Table 3). A combination of TDZ and 2,4-D increased callusing response from 35.0% to 60.0%; however, there was no significant difference in shoot regeneration from the calli compared to results obtained by TDZ alone (Tables 2 and 3). Treatments containing BAP and 2,4-D increased callus induction (from 5.71% to 34.0%) and shoot formation (from 2.86% to 15.0%) from the callus clumps. The efficacy of TDZ together with 2,4-D for shoot induction has been reported in a number of plant species (Zhou et al., 1994; Rugkhla and Jones, 1998; Gairi and Rashid, 2004; Joshi et al., 2008; Amali et al., 2014).

### 3.3. Rooting and acclimatization (hardening)

After generating multiple shoots from the callus clumps, regenerated shoots were successfully rooted on 1/4 strength MS medium (Figures 2F and 2G). This medium was chosen

**Table 3.** Effect of BAP or TDZ in combination with 2,4-D on callus induction and shoot regeneration from leaf base segments of young leaves (3 weeks old) of *Ananas erectifolius*.

| 2,4-D (μM) | BAP (μM) | TDZ (μM) | Frequency of callus induction (%) | Frequency of shoot regeneration (%) |
|---|---|---|---|---|
| 20 | 0 | 0 | 9.00 ± 0.6$^{d}$ | 0.00 ± 0.0$^{e}$ |
| 20 | 2.5 | 0 | 34.00 ± 2.1$^{bc}$ | 15.00 ± 1.0$^{bc}$ |
| 20 | 5 | 0 | 34.00 ± 1.9$^{bc}$ | 15.00 ± 1.0$^{bc}$ |
| 20 | 10 | 0 | 28.00 ± 1.4$^{c}$ | 5.00 ± 0.3$^{d}$ |
| 20 | 20 | 0 | 25.00 ± 0.9$^{c}$ | 0.00 ± 0.0$^{e}$ |
| 20 | 0 | 2.5 | 48.00 ± 2.4$^{ab}$ | 10.00 ± 0.5$^{c}$ |
| 20 | 0 | 5 | 54.00 ± 2.1$^{a}$ | 40.00 ± 1.2$^{a}$ |
| 20 | 0 | 10 | 40.00 ± 1.6$^{b}$ | 20.00 ± 1.0$^{b}$ |
| 20 | 0 | 20 | 60.00 ± 1.9$^{a}$ | 35.00 ± 1.6$^{a}$ |

Frequencies followed by the same lowercase letter in a column are not significantly different by the Scott–Knott test ($P \leq 0.05$). The data represent the mean frequency ± standard error (SE) of 3 independent repeats.

because it was effective for root induction in a preliminary study (data not shown). In addition, using MS medium at a reduced concentration (1/2 or 1/4 strength) increases root production and elongation (Souza et al., 2007). A similar observation was reported in *Ruscus hypoglossum*, in which 1/2 or 1/4 strength MS medium was beneficial for root formation (Dahab et al., 2005). Sikdar et al. (2012) proposed that using MS at reduced concentrations causes nutrient deficiency; as a result, the plantlets produce more roots to increase root surface area in order to absorb the required nutrients. After 30 days in rooting media, the plantlets (~6 cm; Figure 2H) were transferred to plastic pots (20 × 25 cm) filled with commercial compost, and 475 out of 500 plantlets survived (~95%) after acclimatization (Figure 2I).

### 3.4. Influence of pulse treatment on callus and shoot induction

In an attempt to achieve a high frequency of callus induction and subsequent shoot regeneration, pulse treatment with TDZ and 2,4-D was applied to the explants for different periods (5, 10, 15, 20, or 25 days). As a result, the frequency of callus induction and shoot regeneration increased. The highest frequency of callus induction and shoot regeneration was obtained when 10 days of pulse treatment was applied; however, the frequency gradually declined when pulse treatment was extended to more than 10 days (Figures 3 and 4). This implies that applying short-time pulse treatment with growth regulators increased callusing and shooting response of leaf base segments of *A. erectifolius* more than exposing the explants on medium containing growth regulator for a long time. Similarly, Santos (2010) reported a high frequency (70%) of callus induction after 10 days of pulse treatment with NAA and IBA in *Coffea canephora* 'Conilon'. Regarding the use of pulse treatment with growth regulators to enhance callus induction and shoot regeneration, Cruz et al. (1990) explained that applying a pulse with 2,4-D for at least 5 days promoted somatic embryogenesis and shoot induction in *Feijoa sellowiana* Berg. In addition, Guerra et al. (2001) reported that using a pulse with 2,4-D for 2 weeks before transfer to MS medium induced a high number of shoots in *Acca sellowiana*. In the same report, the authors suggested that somatic organogenesis was dependent on factors such as genotype of the explants, type of growth regulator used, and duration of pulse treatment.

The protocol described here for indirect organogenesis using leaf base segments offers a new understanding regarding micropropagation of *A. erectifolius*, because this is the first report addressing callogenesis and shoot regeneration in *A. erectifolius* in a broad concern. Based

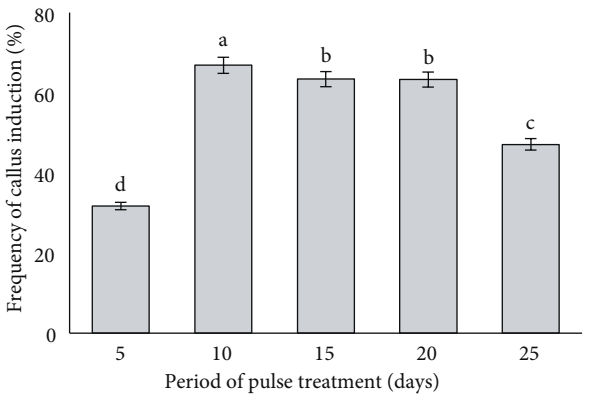


**Figure 3.** Effect of pulse treatment with TDZ and 2,4-D on callus induction in *Ananas erectifolius*. The bar represents the mean frequency ± standard error (SE) of 3 independent repeats.

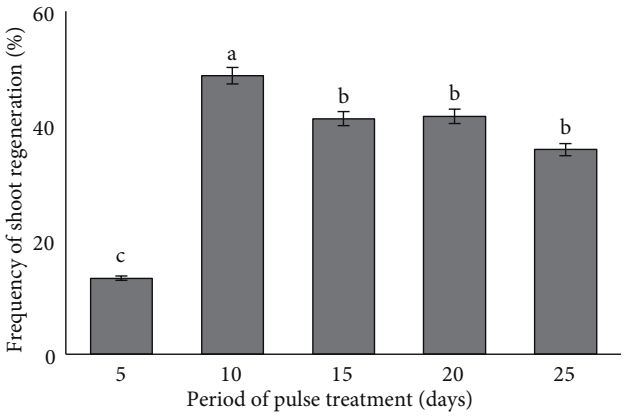


**Figure 4.** Effect of pulse treatment with TDZ and 2,4-D on shoot regeneration in *Ananas erectifolius*. The bar represents the mean frequency ± standard error (SE) of 3 independent repeats.

on the results, we conclude that leaf base segments from young leaves of *A. erectifolius* possess a high amount of meristematic cells since they were the only explants that induced calli as well as shoot regeneration among the three types of explants used. Therefore, we hope that the issues discussed will contribute to large-scale propagation and plant breeding programs in *A. erectifolius* and in other *Ananas* species.

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