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The Sensitivity of Plant Tissue Culture and Plant cell of *Citrullus lanatus* cv. Round Dragon Against BASTA®

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Abstract: Current study provides an efficient screening system for transformed plant of *Citrullus lanatus* cv. Round Dragon harboring *bar* gene. The untransformed 5-day-old cotyledon explants were cultured on the shoot-inducing media supplemented with Basta® (0.2, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}) and without Basta® for 3 weeks and subcultured on fresh shoot-inducing media with the same media composition for another 3 weeks. The shoot growth on the cotyledon decreased, as the Basta® concentration increased. A complete inhibition of shoot growth was observed on growth medium supplemented with 2.0 and 3.0 mg L^{-1} of Basta®, respectively. For *ex vitro* condition, untransformed healthy plant leaves (derived from acclimatized *in vitro* plantlets) were leaf painted with an aqueous solution of Basta® at the concentration of 0.001, 0.01 and 0.1% (v/v) using writing brush. The sensitivity of untransformed plant tissues were evaluated based on tissue browning and necrosis due to herbicidal damage. Healthy plant leaves subjected to leaf painting assay showed serious necrotic within 3 days at the concentration of 0.1% (v/v) of Basta®. An efficient herbicide Basta® selection mode has been established via *in vitro* and *ex vitro* conditions of untransformed *Citrullus lanatus* cv. Round Dragon.

Key words: Herbicide BASTA, leaf painting assay, plant necrosis, Round Dragon, *Citrullus lanatus*

INTRODUCTION

Crop improvement through genetic engineering has become a reality with the successful introduction of agronomical desirable herbicide-resistance trait in various plant species such as in cabbage (Sretenovic-Rajicic *et al*., 2004) orchardgrass (Denchev *et al*., 1997) Norway spruce (Brukhin *et al*., 2000) and sugarbeet (Kishchenko *et al*., 2005). The availability of transgenic herbicide-resistance plant species would improve weed control and increase profitability of the economically important crops (Kishchenko *et al*., 2005). Application of gene transfer techniques using the *bar* gene as a selectable marker has been broadly used in plant species such as sugarcane (Manickavasagam *et al*., 2004) cassava (Sarria *et al*., 2000) legume (Lohar *et al*., 2001) and tobacco (Lutz *et al*., 2001). However, there was only one reported case so far concerning *bar* gene-based *Agrobacterium tumefaciens*-mediated transformation system of *Citrullus lanatus* (Cho *et al*., 2008). The *bar* gene was originally cloned from *Streptomyces hygroscopicus* and has been widely used to engineer

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herbicide-resistant plants (Lea *et al*., 1984; Thompson *et al*., 1987). Stable integration of *bar* gene into crop genome via *Agrobacterium tumefaciens*-mediated transformation system has yielded plants resistant to the herbicide Basta®. Basta® is a non-selective herbicide being used in agriculture sectors made up of phosphinothricin (PPT), an analogue of L-glutamic acid and two L-alanine residues (Keller *et al*., 1997). Basta® acts as a potent herbicide as its active ingredient, PPT interferes with amino acid synthesis through inhibition of glutamine synthetase involved in the detoxification of ammonia in plants (De Block *et al*., 1987). Inhibition of glutamine synthetase by PPT causes a rapid build up of intracellular ammonia levels which associated with disruption of chloroplasts structure results in inhibition of photosynthesis and plant cell death (Tachibana *et al*., 1986; Lutz *et al*., 2001). Thus, a stable introduction of *bar* gene that encodes the enzyme phosphinothricin acetyltransferase (PAT) into the plant genome detoxifies PPT by acetylation of the free ammonia group, thereby neutralizing its toxic effect on plant cells (Thompson *et al*., 1987; Strauch *et al*., 1988). In addition, study indicates that *Agrobacterium tumefaciens*-mediated transformation using the *bar* gene is possible to produce a transgenic plants that confers resistance to herbicide Basta® (Qing *et al*., 2000; Yoon *et al*., 2002; Choi *et al*., 2004).

In this study, the use of herbicide Basta® as a selective agent was described. The main objective was to establish a selection method between transgenic and non-transgenic *Citrullus lanatus* via plant tissue culture and leaf painting assays. This would help to select the transgenic *Citrullus lanatus* that express *bar* gene in plant tissue.

MATERIALS AND METHODS

In vitro Basta® Sensitivity Test

In vitro sensitivity test for commercial herbicide Basta® (Bayer Crop Science) was carried out using 5-day-old cotyledon explants of *Citrullus lanatus* cv. Round Dragon. The cotyledon explants were excised 1-2 mm above the point of attachment to the hypocotyls as described by Dong and Jia (1991). The distal portions of the cotyledons were discarded and the proximal region was used as explant segments.

The proximal region was cultured abaxial side down in 90×15 mm plastic petri dishes that contained MS shoot regeneration medium supplemented with 2.3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 3.2 g L^{-1} Phytagel and various concentration of commercial herbicide Basta® (0.2, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}). The control was maintained by culturing the cotyledon explants on shoot induction medium without Basta®.

Cell growth was maintained under a 16 h photoperiod of 12.16 μmol/m^2/sec from cool white fluorescent lamps at 25±1°C in a tissue culture chamber for 3 weeks. The explants were subcultured to fresh medium with the same media composition every 3 weeks and stop after 6 weeks. Each experiment was repeated three times with 30 cotyledon segments per treatment and 5 explants per Petri dish. After 6 weeks, the frequency of survived explants with shoots and number of shoots per explant were numerated by stereomicroscope. At this time, the sensitivity of cotyledon explants was evaluated based on tissue browning and necrosis.

Ex vitro Assay for Basta® Sensitivity

Ex vitro assay to test Basta® sensitivity were conducted according to Akama *et al*. (1995). Cotyledon of *Citrullus lanatus* was grown in plant tissue culture growth medium as earlier described. The rooted plantlets from plant tissue culture medium (about 3-5 cm with >1 roots) were transferred to pots and transparent plastic covers were placed over the plantlets. During 1 week, the humidity level was maintained under a 16 h photoperiod of

12.16 μmol/m^2/sec from cool white fluorescent lamps at 25±1°C in a tissue culture chamber. After 1 week, the plastic covers were removed gradually over 3 days and watered with nutrient solution to allow growth.

After acclimatization period (about 1 month), the untransformed plants were selected based on Basta® sensitivity via leaf painting assay. The plants were tested by painting a few leaves on the upper surface using writing brush with an aqueous solution of Basta® originally containing 13.5% (w/w) glufosinate ammonium (Bayer Crop Science) diluted to 0.001, 0.01 and 0.1% (v/v). The leaves were observed daily and scored based on herbicide damage of untransformed plants.

Statistical Analysis

Data were analyzed using one-way Analysis of Variance (ANOVA) to compare the means for more than one treatment. Variation among treatment means was analyzed using Tukey's Honestly Significant Difference Test (HSD value) and the significance were determined at the $p<0.05$ level (Jackson and McLean, 1998). Statistical analysis was performed using SPSS for Windows software (SPSS Windows Version 15). Data are presented as means and standard errors.

RESULTS

Effect of Basta® on *in vitro* Shoot Regeneration

The experiment was carried out to test the sensitivity of untransformed shoots towards commercial herbicide Basta® in order to find out the appropriate concentration of selection agent on *in vitro* transformed shoots regeneration. According to Table 1 at 2 mg L^{-1} of Basta® led to complete inhibition of regeneration of shoot on MS medium. In control experiment, after 6 weeks, 93% adventitious shoots were regenerated at the proximal region of cotyledon. Shoots regenerated per responding explant drops dramatically on MS medium supplemented with 0.2 mg and 0.5 mg L^{-1} of Basta® compared to explant regenerated on MS medium without Basta®. At a concentration of 0.2 mg L^{-1} of Basta®, about 77% of cotyledon explants produced callus after 1 week followed by shoot bud formation at week 3. The explants were subcultured again to a fresh MS shoot regeneration medium supplemented with 0.2 mg L^{-1} of Basta® after 3 weeks and were capable to induce normal shoots even at low mean number of shoots per explant (Fig. 1a-f). The frequency of shoot regeneration on cotyledon explants decreased to 50% in the concentration of 0.5 mg L^{-1} Basta®. The shoots remained green at week 6 but grew slowly and smaller than control.

The cotyledon explants exposed with Basta® at 1 mg L^{-1} turned yellow with necrosis within 3 weeks. About 23% of explants survived from necrosis as well as formed callus and enable to produce shoot buds at week 6. Medium supplemented with 2 and 3 mg L^{-1} of Basta®, showed a complete necrosis occurred at cotyledon explants (with yellow turned to

Table 1: Effect of *in vitro* shoot regeneration on MS medium supplemented with various concentration of Basta®

Concentration of Basta® (mg L^{-1})	Frequency of explant resistant to Basta® (%)	No. of shoots per explant resistant to Basta®
0.0	93	19.93±0.99^a
0.2	77	5.80±1.00^b
0.5	50	2.13±0.54^c
1.0	23	0.57±0.06^c
2.0	0	0.0
3.0	0	0.0

Values within a column followed by different superscripted letters are significantly different at the $p<0.05$ level

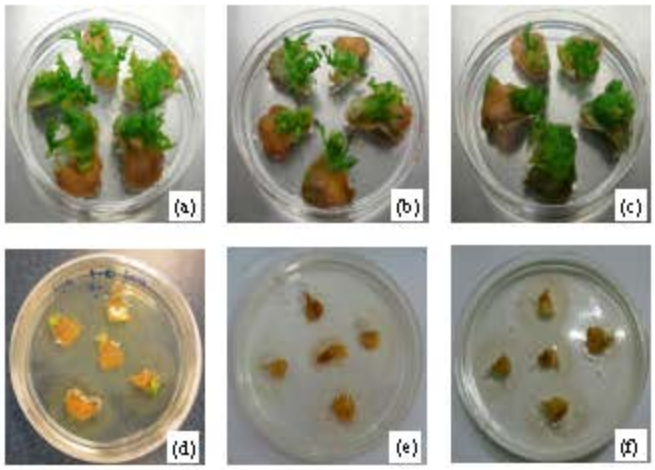

Fig. 1: Preliminary sensitivity test of Basta®. (a) control explants (MS medium without Basta®), (b) shoot regeneration on MS medium with 0.2 mg L^{-1} Basta®, (c) shoot induction on MS medium supplemented 0.5 mg L^{-1} Basta®, (d) necrosis occurred within 3 weeks but shoot bud formed at week 6 with 1.0 mg L^{-1} Basta® and (E-F) complete necrosis occurred on cotyledon explants supplied with 2 and 3 mg L^{-1} Basta®, respectively

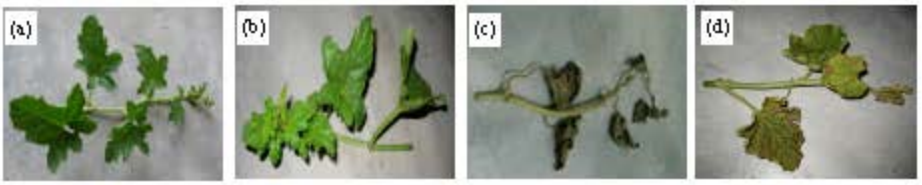

Fig. 2: Leaf painting of *Citrullus lanatus* exposed with Basta®. Untransformed plants were leaf painted with aqueous solution of diluted Basta® at (a) 0%, (b) 0.001% (c) 0.1% and (d) 0.01%, respectively. The observations were taken after day 3

brown) within 2 week and no callus or shoot bud was initiated from any cotyledon explants. Since, there were no significant difference was detected between the concentration of 2 and 3 mg L^{-1} of Basta® used, therefore, 2 mg L^{-1} of Basta® was chosen as the selective concentration to be used for selecting transgenic plants.

Leaf Painting Assay Using Herbicide Basta®

Herbicide leaf painting assay were conducted to assess the sensitivity of untransformed plants of *Citrullus lanatus* towards commercial herbicide Basta®. A 1 month old of untransformed healthy plant leaves were selected. Different concentrations of Basta® were applied using a writing brush to the upper surface of the selected leaves of *Citrullus lanatus*. Untransformed plants treated with varying concentration of Basta® showed different level of sensitivity (Fig. 2a-d).

The effect of commercial herbicide Basta® sensitivity on untransformed plants was observed within 3 days. Figure 2 showed untransformed plants treated with 0.001% (v/v) of

Basta® remain green after 3 days and showed no symptoms of herbicidal damaged. In contrast, plants exposed to Basta® at the concentration of 0.01% (v/v) started to turn yellow, bleached and showed necrotic within 3 days. However, the plants leaf painted with 0.1% (v/v) of Basta® died within 3 days period. This result indicated that leaf painting with commercial herbicide Basta® at the concentration of 0.1% (v/v) yielded high sensitivity to *Citrullus lanatus*.

DISCUSSION

Preliminary sensitivity test of the commercial herbicide Basta® were performed, prior to the gene transfer studies on non-transgenic *Citrullus lanatus* to determine the best concentration of Basta® to screen and to select the transgenic plants harboring the Basta resistance gene (*bar*). Current study is useful for future research to establish *Citrullus lanatus* that confer a resistance to herbicide Basta®. A highly efficient selective condition towards herbicide Basta® from the 5-day-old cotyledon explants has been evaluated by culturing the explants on shoot induction medium supplemented with various concentrations of Basta®.

Cotyledon explants treated with 0.5 mg L^{-1} of Basta® enable to induce shoots but grew slowly and smaller compared to the shoots from the control. Under this non-selective condition, adventitious shoot appeared at the proximal region of the cotyledon explants indicating tissue culture technique used here similar with those Han *et al.* (2005) and Dang and Wei (2007). This finding supports previous research that claimed cotyledon explants exposed with 0.5 mg L^{-1} of Basta® eventually survived and formed adventitious shoots in bottle gourd (Han *et al*., 2005) and in leguminous tree (Vengadesan *et al*., 2006). However, it was not consistent with cotyledon explants of Perilla were unable to form any callus or shoots when treated with 0.5 mg L^{-1} of PPT (Kim *et al*., 2004). Thus, it can be concluded that the concentration of Basta® required for the selection highly dependent on the plant species.

Cotyledon explants treated with 1.0 mg L^{-1} of Basta® turned yellow and partially survived with necrosis. These findings confirmed the results of other research that the embryonic tip system of soybean died when treated with 1.0 mg L^{-1} of PPT (Dang and Wei, 2007). A severe necrotic of cotyledon explants was observed on the shoot induction medium supplemented with 2 and 3 mg L^{-1} of Basta®. These findings agreed with Vengadesan *et al*., (2006) that showed PPT above 1.5 mg L^{-1} led to complete inhibition of shoot regeneration. Moreover, this findings support the idea raised in other research that 2 mg L^{-1} of PPT in *Perilla frutescens* and 3 mg L^{-1} of bialaphos in *Dactylis glomerata* (Denchev *et al*., 1997) caused the untransformed plant died with necrosis.

It has been reported that the concentration of PPT was gradually increased during the selection to prevent any escapes in soybean (Dang and Wei, 2007). It may be beneficial if the primary transgenic shoots cultured on the medium supplemented with 2 mg L^{-1} of Basta® and the secondary transgenic shoots from primary shoots cultured on the medium supplemented with 3 mg L^{-1} of Basta® in order to increase the selection pressure. However, the current study did not support the idea. Thus, the transformed shoots were subjected to the same level of selection pressure to eliminate possible chimeric plants and escapes. In addition, it has been suggested that exposure of the cotyledon explants on a high selection pressure for a longer period will eliminate many non-transformants (Dang and Wei, 2007).

Based on the preliminary experiment, 2 mg L^{-1} of Basta® was chosen as the minimal and stringent concentration for selection of transgenic plants similar to bottle gourd (Han *et al*.,

2005). The concentration of selecting agent of Basta® for transgenic *Citrullus lanatus* recovery in this study were lower than those used in other plants such as rice at 4 mg L^{-1} (Cao *et al*., 1992), bean at 5 mg L^{-1} (Russel *et al*., 1993) sugarcane at 5 mg L^{-1} (Manickavasagam *et al*., 2004) and woody plants at 10 mg L^{-1} (Choi *et al*., 2004). However, continuous culture of regenerating explants on selection medium supplemented with 2 mg L^{-1} of Basta® inhibit the number of shoots regenerating from the explant and were used for selecting transgenic *Citrullus lanatus* in subsequent experiments.

Leaf painting assay assessed the sensitivity of the grown untransformed plants towards commercial herbicide Basta®. Control experiment plants treated with 0.001% (v/v) of Basta® continued to grow and no changes were observed on the physical state of the plants. In contrast, plants treated with 0.01% (v/v) bleached caused necrotic after a single painting. It seems possible that these results are due to foliar damage of the plants which were sensitive to Basta® (Lohar *et al*., 2001).

Furthermore, a plant exposed with 0.1% (v/v) of Basta® bleached, shrank, died with severe necrosis within 3 days. This findings support previous research that untransformed cassava plants showed necrosis after a single spraying (Sarria *et al*., 2000). In addition, application of herbicide on plants caused the photosynthesis slowed down within 2-4 h. After spraying, the plants became yellow and died within 2-5 days (Yoon *et al*., 2002). Thus, the expression level of untransformed *Citrullus lanatus* leaf painted with Basta® at 0.1% (v/v) was sufficient to obtain a transformed plants resistance to herbicide Basta®. Han *et al*. (2005) also reported the similar result on the bottle gourd by spraying with a 0.1% (v/v) of Basta® solution.

In conclusion, the current study demonstrates the development of an efficient selection protocol in order to screen and to produce a transgenic plant that confers a resistance to herbicide Basta®. Preliminary study on Basta ®sensitivity tests suggested that selection medium supplemented with 2.0 mg L^{-1} of Basta® and leaf painting assay applied with 0.1% (v/v) Basta® yielded optimum selection condition for transgenic plants with low number of escapes of non-transgenic plants.

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