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Analysis of the Inhibitory Concentration of Ammonium Glufosinate in Cotyledons Explants of Tomato Plants (*Solanum lycopersicon*)

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Abstract: In recent years, biotechnology has expanded the use of plants by introducing important genes that need to be expressed in large amounts or that contain medicinal properties. Due to this, systems to distinguish the transformed cells from the non-transformed cells are indispensable. Selectable marker genes are important tools for plant genetic transformation. In transformation process, the selection marker that usually determines herbicide or antibiotics resistance is introduced together with the gene of interest and the transgenic condition is revealed by expression of the selection marker, allowing the transformed cells or tissues to survive on medium containing the selective agent. Thus, knowledge about the relative tolerance of plant tissues to selective agents facilitates *in vitro* cultivation and the selection of species transformed. Cotyledon explants of *Solanum lycopersicon* were transferred to regeneration medium containing different concentrations of the herbicide ammonium glufosinate, called Phosphinothricin (PPT) and analyzed for their regenerative response. The results indicate a PPT's concentration of 0.5 mg L^{-1} to be used in the selection of transformed plants.

Key words: Tomato, selective agent, genetic transformation, phosphinothricin, biotechnology

INTRODUCTION

In recent decades, the development of plant-based technologies have improved knowledge and understanding of plant molecular biology, which allows them to be modified for successful applications in fields such as agriculture, medicine and industry. Plant biotechnology is based on the transfer, integration and expression of defined genes into plant cells, which can generate transformed plants. The efficiency of the process is not high and only a fraction of the cells are successfully transformed. Consequently, systems to distinguish the transformed cells from the non-transformed cells are indispensable. For this reason, selectable marker genes are important tools for plant genetic transformation (Sundar and Sakthivel, 2008).

In transformation process, the selection marker that usually determines herbicide or antibiotics resistance is introduced together with the gene of interest and the transgenic condition is revealed by expression of the selection marker, allowing the transformed cells or tissues to survive on medium containing the selective agent, while non-transformed cells and tissues die (Nap *et al*., 1992). The cell that comprises the selectable gene will express the gene of interest, once both genes are carried together into the nucleus of the cell. Different selectable marker genes were used for transgenic plant research. The antibiotics kanamycin and hygromycin and the herbicide phosphinothricin were widely used as selection agents due their efficiency, availability and applicability (Sundar and Sakthivel, 2008).

The herbicide phosphinothricin (PPT) or ammonium glufosinate, is analogous to glutamate, the substrate of glutamate synthetase. This enzyme is involved in the conversion of glutamate to glutamine, removing the toxic ammonia from the cell. Therefore, it has an essential role in the regulation of nitrogen metabolism and ammonia assimilation. The enzyme phosphinothricin N-acetyltransferase (PAT) encoded by gene *bar*, the resistance gene from *Streptomyces hygroscopicus*, detoxifies PPT by acetylation using acetyl-coenzyme A as cofactor (De Block *et al*., 1987; Dekeyser *et al*., 1989; Wilmink and Dons, 1993; D'Haullin *et al*., 1992). The *bar* gene has been widely used as an effective selectable marker in many crop species, including maize (Gordon-Kamm *et al*., 1990), rice (Cao *et al*., 1992), wheat (Weeks *et al*., 1993), sweet potato (Yi *et al*., 2007), grapevine (Jardak-Jamoussi *et al*., 2008).

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The present study aimed to evaluate the concentration of the herbicide ammonium glufosinate to be used as selective marker in the genetic transformation of explants of tomato (*Solanum lycopersicon*).

MATERIALS AND METHODS

This study was conducted in plant biology and general biology departments of the Federal University of Viçosa, Minas Gerais State, Brazil in the period from January to September 2011.

Explant preparation: *Solanum lycopersicon* (Moneymaker cultivar) seeds were courtesy from Laboratory of Plant Tissue Culture-Bioagro/ Federal University of Viçosa, Minas Gerais. The tomato seeds were sterilized by immersion in 70% ethanol for 1 min and 2.5% (v/v) sodium hypochlorite solution for 20 min. Then, the seeds were washed in sterile distilled water and immersed in 1% sodium hypochlorite solution for 12 h. Finally, the seeds were washed four times in sterile distilled water and dried on sterile filter paper. The seeds were grown in Murashige-Skoog (MS) solid medium (Murashige and Skoog, 1962) containing half of salt concentration (100 mg L^{-1} i-inositol, 2 mg L^{-1} thiamine.HCl, 0.5 mg L^{-1} pyridoxyne.HCl, 0.5 mg L^{-1} niacin, 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.8). Seedlings were grown at 27±2°C, 24-36 μmol m^{-2} sec^{-1} of irradiance, 8 h dark and 16 h light. Cotyledons of 8-day-old plants, counted from the radicle emission, were removed and cut into two fragments.

Sensitivity of cotyledon explants to ammonium glufosinate: Two experiments were conducted to examine the inhibitory concentration of ammonium glufosinate in tomato explants. Briefly, 10 cotyledon pieces were placed upside down in 90×15 mm Petri dishes containing regeneration medium and ammonium glufosinate (Finale, Bayer CropScience AG, Germany) at 0, 0.1, 0.2, 0.3, 0.4 or 0.5 mg L^{-1} in the first experiment and, in the second experiment, at 0, 0.4, 0.7, 1.0, 1.3 or 1.6 mg L^{-1}. Explants were incubated at 27±2°C, 24-36 μmol m^{-2} sec^{-1} of irradiance, 8 h dark and 16 h light, with medium maintenance from each 15 days and observed for 60 days. Each treatment in the first experiment was conducted using 10 explants, in triplicate (total: 30 explants per treatment) and in the second experiment, using 10 explants in five replicates (total: 50 explants per treatment).

Data analysis: The experimental design was randomized. In the descriptive analysis was performed to absolute and relative frequency.

RESULTS AND DISCUSSION

After germination, the cotyledon explants were transferred to regeneration media containing concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg L^{-1} glufosinate ammonium. The explants were transferred to new MS media with their treatments and analyzed for callus formation each fifteen days. After 60 days, the explants cultured at concentrations below 0.4 mg L^{-1} ammonium glufosinate produced a large amount of callus, demonstrating that under these conditions, the explants are tolerant to this selective agent and therefore these concentration values cannot be used for selection during transformation (Fig. 1). According to Nabors *et al*. (1983), different types of callus can be induced in different medium and they can differ by morphological characteristics and by the potential of plant regeneration. The callus, called embryogenic, presents the formation of small somatic embryos capable of regenerating whole plants with roots and shoots, since they are bipolar. So, we can infer that the minimum inhibitory concentration for a selective agent in transformation process must not cause necrosis (death) of the explants and also at the same time reduce the regeneration of the tissue, which can be observed by reducing the formation of callus.

The ammonium glufosinate at concentration of 0.5 mg L^{-1} significantly reduced the regeneration of calli when compared to control. At this concentration occurred the inhibition of 66% of the regeneration of explants without causing the death of the same (Fig. 1).

A second test was performed to analyze the effects of ammonium glufosinate in higher concentrations. After new germination of the seeds, the cotyledon explants were transferred to regeneration media containing concentrations of 0, 0.4, 0.7, 1.0, 1.3 and 1.6 mg L^{-1} ammonium glufosinate. It was found in the explants used the survival rate decreased with increasing concentration of the herbicide and from 0.7 mg L^{-1} of the selective agent there was necrosis was the same (Fig. 2), indicating that the concentration value of 0.5 mg L^{-1} of ammonium glufosinate is most appropriate for use being this condition necessary for the success of the selection of the transformed plants (*Solanum lycopersicon*).

Subsequently, a death curve was drawn after 60 days, to assist in determining the best inhibitory concentration of the herbicide to be used for selection of plants during transformation.

To optimize the *in vitro* selection system in tomatoes (*Solanum lycopersicon*), we analyzed the effects of cotyledon explants regeneration in different concentrations of ammonium glufosinate, since in

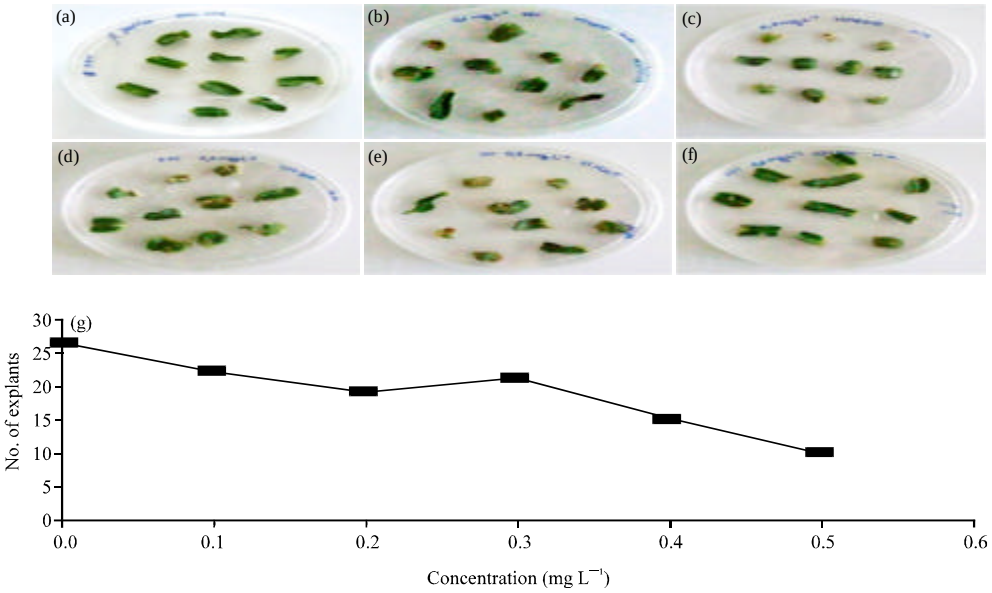

Fig. 1(a-g): Sensitivity of tomato explants in regeneration medium at different concentrations of ammonium glufosinate. Each plate contains 10 tomato cotyledon explants and represents three independent experiments, (a) Control, (b) 0.1 mg L^{-1}, (c) 0.2 mg L^{-1}, (d) 0.3 mg L^{-1}, (e) 0.4 mg L^{-1}, (f) 0.5 mg L^{-1} and (g) Graphic representation

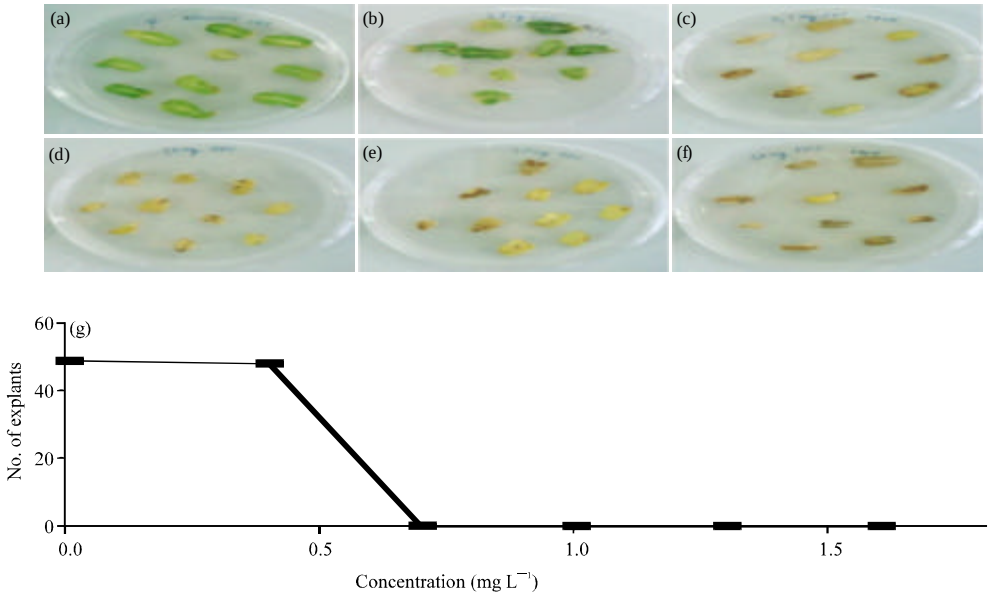

Fig. 2(a-g): Sensitivity of tomato explants in regeneration medium at different concentrations of ammonium glufosinate. Each plate contains 10 explants and each concentration of herbicide was analyzed in five replicates, (a) Control, (b) 0.4 mg L^{-1}, (c) 0.7 mg L^{-1}, (d) 1.0 mg L^{-1}, (e) 1.3 mg L^{-1}, (f) 1.6 mg L^{-1} and (g) Graphic representation

literature there are little information about the use of this selective agent in transformation of tomato (Hussain *et al*., 2008).

In recent years, biotechnology expanded the use of plants by introducing genes which have genes to be expressed in large amounts or which contain medicinal

properties. In plants, mass production of interest proteins becomes commercially cheaper. These advantages have allowed the expression of a wide variety of proteins for the prevention, diagnosis and therapy (Blais and Altosaar, 2006; Streatfield, 2006).

In Brazil, there are few studies in the field of *in vitro* regeneration and genetic transformation of tomato cultivars and little is known about the regenerative capacity of the main Brazilian cultivars (Fari *et al*., 2000).

According to Hansen and Wright (1999), to obtain a successful transformation process it is necessary to consider some requirements such as: the target tissue of the transformation should have the competence to be propagated or regenerated; agents that select transgenic tissues should be used; the transformation process must be simple, efficient and low-cost. In view of this, we can say that this system of selection using *in vitro* ammonium glufosinate is of great value as a tool to assist in the selection process of transformed plants. The results obtained are consistent with Lilge *et al*. (2003) reports that the importance of this selection system in rice and in accordance with other studies that used this selective agent in transformation processes in plants of the genus *Cucumis* (Vasudevan *et al*., 2007), *Daucus* (Chen and Punja, 2002), *Arabidopsis* (Akama *et al*., 1995), *Nymphaea* (Pigeaire *et al*., 1997), *Medicago* (D'Hallun *et al*., 1990), *Pisum* (Schroeder *et al*., 1993), *Glycine* (Zhang *et al*., 1999), *Saccharum* (Manickavasagam *et al*., 2004) and *Carica* (Cabrera-Ponce *et al*., 1995), obtaining transgenic plans with genes of interest. The conditions set out in this study for *in vitro* culture and genetic transformation of tomato plants can contribute to the development of new cultivars containing genes of interest. It is important to determine the dose of the herbicide to be used in order to prevent the occurrence of false positives without interfering at the same time, in their regenerative potential, so that the greatest number of transgenic shoots is selected (Brasileiro and Lacorte, 1998).

CONCLUSION

The analysis of the regeneration of cotyledon explants of tomato plant indicate the concentration of 0.5 mg L^{-1} ammonium glufosinate ideal for plant selection during the transformation processes. This concentration can be used for selection of transformed plants in future studies of gene transfer in *Solanum lycopersicon*.

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