Evaluation of glyphosate and an organic mercuric compound, phenyl mercuric acetate, as selective agents for peppermint (*Mentha piperita L.*) transformation

Shirin Bahrampour¹, Bahram baghban², Majid Shokrpour³*

1- MSc. in Agricultural Biotechnology
2- Dept. of Agronomy, Faculty of Agriculture, University of Tabriz
3- Dept. of Horticulture, College of Agriculture and Natural resources, University of Tehran

*Corresponding Author Email: shokrpour@ut.ac.ir

Abstract

Efficiency of transformation methods is due to precise, simple and quick identification of transformed and non-transformed tissues. To determine a suitable selective system has an important role in success of plant transformation. In this research, the effect of glyphosate and phenyl mercuric acetate as selective agents, were studied. Plant samples were provided from field, greenhouse and in vitro cultures. Different tissues as shoots and leaf disks exposed to different concentrations of the agents. Results of analysis of variance (ANOVA) showed a significant concentration×day interaction at 0.01 level. Effect of the agents on non-transformed peppermint tissues were appeared as necrosis in tissues and death at last. Mean comparison results for PMA showed that 2 to 4 concentrations would be caused plant death over 16 days, in shoot and leaf disk explants, respectively. This result was obtained in the concentration of 1.5 ppm during 10 days. Glyphosate concentration of 10000 and 2000 ppm caused necrosis and death of shoot and leaf samples during 2-3 and 6-9 days, respectively. Influence of this agent on leaf disks had different pattern in the field and greenhouse conditions. In conclusion, results of this study revealed that in attention to research aim, each of these agents can be applied as selective agent for peppermint transformation and the above critical doses may be used in optimum selecting non-transformed tissues.

Keywords: glyphosate, peppermint, phenyl mercuric acetate, selective agent

Introduction

During thousands of years, medicinal plants were the most important source of valuable drugs in the most societies over the world. According to World Health Organization (WHO) report, about 80 percent of the world population applies traditional herbal medicine for healing purposes (Tripathi and Tripathi, 2003). Growing interesting herbal drugs and the resistance to use chemical drugs because of their side effects, have been led to run extensive and effective researches in improving quality and quantity of active ingredients in medicinal plants.

Peppermint, *Mentha piperita* L., has significant economical and pharmaceutical potentials (Omidbaigi, 1997). Since the commercial varieties and relative species of peppermint have the sterile flowers and different ploidy levels, respectively, application of traditional breeding methods are restricted (Kransianski et al, 1999; Verones et al, 2001). In the other words, effect of plant biotechnology and molecular breeding in elevation of yield some agricultural products and using them for making the plants with new attributes are clear (Azadi and Bagheri, 2003). So, in order to increase the amount of peppermint essential oil, it can be used biotechnological techniques. Successful application of transformation, an important biotechnology approach, needs to make a special and appropriate protocol. Since, a substantial challenge in this regard is need to introduction of new and safe selective systems, study of plant tissues response to selective agents is
the first step. Selective agents includesome compounds that act during various mechanisms inside the plant cells and cause to death, stop or enhance of growth and normal activities of them. Only cells that can resist stopping effect of the agents, would survive and the others would die, eventually sometimes one selective system is not sufficient for all purposes, so it needs to several systems (Miki & McHugh, 2004). Also, response of different plants to type of selective system and its amount and dose may be different. Thus the need to specific protocols for each plant is quite obvious. The protocols determine type and amount of selective agents to distinguish transformed and non-transformed cells efficiently. Among the agents can point out glyphosate that is a herbicide with a broad range of effect and is active compound of commercial Round-up. It stopsessensatinal process of stop photosynthesis (Farsi and Zolala, 2003). This agent as an inhibitor may block an important metabolic pathway that is catalyzed by EPSP synthase, a plastid enzyme, in plants (Ye et al., 2001). In some plant species, glyphosate resistant lines have been selected by various experimental methods. Resistant cell lines have been isolated in carrot, tobacco, tomato, pea, Catharanthus rosas and Corydalis sepervirens cell suspension and or callus cultures. Organic compounds of mercury such as Phenyl mercuric acetate (PMA) can be applied as selective agents. These influence laticoid membranes of chloroplast and photosynthesis which stop electron transfer, oxygen production, Hill reaction and phosphotransferase and turn off chlorophyll fluorescence in photosystem II (Ruiz et al., 2003). Since the selective systems playmain role in gene transfer protocols and in attention to importance of peppermint as a medicinal plant, this research was conducted to evaluate effects of glyphosate and PMA on peppermint and determine most efficient dose of them to kill non-transformed tissues.

**Material and Methods**

Plant material: plant samples were collected from peppermint branches grown in greenhouse. As the *in vitro* may show different response to selective agents, thus the plant samples from greenhouse were sterilized and were *in vitro* cultured.

Treatments: Samples of branches, leaves and leaf discs were used to examine the effect of glyphosate and PMA on peppermint. Suspension MS media containing PMA with 0, 2, 4, 6, 8 and 10 ppm concentrations and, for more precise evaluation, 0, 0.5, 1, 1.5, 2, 2.5 and 3 ppm concentrations were made. The different samples with same sizes in each type were put in the media. To obtain the optimal concentration and the effect trend of glyphosate on peppermint, concentrations of 0, 200, 2000, 10000 and 20000 ppm were prepared. Also to assess glyphosate effect on leaf discs explants, the dose of 0, 200, 400, 600, 800 and 1000 ppm were applied. Effects of PMA and glyphosate on the explants as discolored tissues were daily recorded.

Statistical analysis: In order to record changing trends in explants exposed to the selective agents, their images were daily taken by digital camera, Nikon corp., Coolpix 6 model. At the end of the experiment, image samples were rated based on the range of the changes. The rating for leaf samples were assigned from 1 to 5 from the healthy to the fully affected plants. For the branch explants, the rates were 1 to 10 as the broad range of the changes. Analysis of variance was done as split plot in time based on completely randomized design with two replications. Mean comparisons were performed for significant sources by Tukey method at 5% probability level.

**Results and discussion**

Significant differences were obtained for all sources of variances at 1% level. Trend of different concentrations of PMA-induced changes has been displayed in figures 1 to 6 days. Regard to branch explants, effect of dose levels of 0 to 10 ppm during 16 days displayed increasing slope for 4 to 10 ppm along to going days and reached to maximum killing effect at 12th day (fig. 1). While the fatality effect of 2 ppm elevated from the fifth day that the effect was 80% after the day of 16. Thus, it seems that no need to applying the doses more than 4 ppm can be achieved favorable results by lower doses. This was verified by results of the doses of 0 to 3 ppm (fig. 2). The leaf explants from greenhouse (fig. 3) and *in vitro* (fig. 4) were faced to full death at 3rd day by dose of 3 ppm of PMA. Rapid responseof *in vitro* leaf explants in low doses (1.5-2.5 ppm) was because of their fragile and sensitive tissues. Totally, it may be said that the dose of 1.5 ppm of PMA was appropriate to select different non-transformed leaf samples. While for obtaining rapid response needs to apply the dose of 3 ppm. The *in vitro* leaf disc explants were too sensitive to all the studied PMA doses so that they were killed at 3rd day. While the greenhouse leaf disc explants were more resistant and the doses of higher than 4 ppm can be caused to full death of tissues. Therefore it may be proposed that the dose of 4 ppm was effective to extinct different leaf discs over 3 days. Ruiz et al. (2003) produced resistant tobacco plants to organic mercury compounds including PMA by chloroplast transformation using genes of *mer A* and *mer B*. Comparison of transformed and non-transformed plants in doses of 0 to 200 μM (approximately 0 to 70 ppm) displayed that non-transformed plants severely suffered
after 14 days while transgenic plants remained healthy and alive. Rugh et al (1996) produced mercury resistant transgenic Arabidopsis using modified bacterial merA gene. The seeds of the transgenic plants well germinated and grew at concentrations of 50 and 100μM mercury chloride while the non-transgenic plants died in the doses.

Figure 1. Change trends of peppermint branch explants exposed to PMA doses of 0 to 10 ppm

Figure 2. Change trends of peppermint branch explants exposed to PMA doses of 0 to 3 ppm

Figure 3. Change trends of peppermint leaf explants from greenhouse exposed to PMA doses of 0 to 3 ppm

Figure 4. Change trends of peppermint leaf explants from in vitro exposed to PMA doses of 0 to 3 ppm

The changes in the branch and leaf explants under different concentrations of glyphosate were evaluated as a percentage of survival. On the 3rd day, peppermint branch explants died in 10000 and 20000 ppm concentrations. There was no significantly different between the two concentrations. No significant
difference observed among control and 200 ppm doses (Fig.5 - A). The greenhouse leaf explants completely destroyed on the 2nd day in doses of 10000 and 20000 ppm. Like branch explants, it was not found any effect using dose of 200 ppm (Fig.5 - B). Glyphosate effect on leaf disc explants from greenhouse showed that higher concentrations of 400 ppm on the second day caused to the death of the explants. On the 7th day, the discs displayed completely necrosis. The results appeared that to destroy and kill the branch and leaf explants of peppermint must be applied the doses of more than 200 ppm.

![Figure 5. Glyphosate fatal effect trends on branch (A) and leaf (B) explants](image)

Ye et al (2001) were able to produce glyphosate tolerant transgenic tobacco. They measured seedling whitening (discoloring) to evaluate the maternal inheritance of resistance to glyphosate. Thus the seeds derived from selfing of plastid transformants and crosses among transformants and wild type plants were examined on MS medium containing 200 µM glyphosate. Glyphosate caused to fully discoloring of wild type seedlings, but had no effect on germination frequency, while the transgenic seedlings were completely green. However, the resistance was found just when the transplastomic plant was considered as mother plant. Daniell et al (1999) sprayed same volumes of different doses (0.5 to 5 mM) of glyphosate on control and transformed plants on two weeks after germination. Non-transgenic control plants were highly sensitive to glyphosate and destroyed within 7 days even at concentration of 0.5 mM. But the transgenic plants even survived the concentration of 5 mM. Since the glyphosate is a general herbicide it may be pointed that if the glyphosate resistant plants were produced, not only it can be used as selective agent but also needs to use special herbicides and operations to combat weeds will be lost. It is not only economically profitable but also increases the quality of essential oils because of removing weeds.

References