Abstract: Production costs of F1 seeds of Petunia hybrida can be decreased by using sterile component. Cytoplasmic male sterility (CMS), located in mitochondria, is transferred either by back crossing or by protoplast fusion. Asymmetric protoplast fusion represents the most effective way. Protoplast nucleus of one component (donor of CMS) must be destroyed by UV radiation. Protoplast cytoplasm of the second component must be destroyed by metabolic inhibitors. Asymmetrically fused protoplast possesses CMS from donor and rest of the features of fertile component. The aim of this experiment was to determine the amount of UV radiation (in seconds) needed for destruction of protoplast nucleus. Optimal UV radiation, based on results from 3 different Petunia hybrida genotypes is 510 µW/cm² for a duration of 300 seconds.

Key Words: Petunia hybrida, protoplast, cytoplasmic male sterility, UV radiation

INTRODUCTION

Petunia hybrida is among the most popular annual ornamental plants worldwide, so many breeding and production companies put a strong emphasis on reducing production costs and introducing new varieties with improved features, esthetic or growing performance (Anderson 2007). Majority of current assortment are F1 hybrids or varieties multiplied by cuttings (Gerats and Strommer 2009). Production costs of F1 seeds are high due to manual emasculation and pollination of maternal component with a pollen collected from paternal component (Sink 1984). Promising way how to decrease the costs is, besides production relocation to developing countries, inducting cytoplasmic male sterility (CMS) to the maternal component, so functional pollen is not produced. Sterile component does not require emasculation that must occur otherwise every day and represents about 40% of all labor costs.

Transfer of CMS into maternal component is possible via 2 methods, back crossing and protoplast fusion. Back crossing method is used in breeding programs for more than 50 years, but the main drawbacks are: long duration and impossibility to apply it to vegetative components. To transfer CMS by back crossing takes at least 5 or 6 generations, when donor of CMS must be crossed with a fertile component (Gaus 2002).

Second, more promising method, is protoplast fusion, that is used primarily by researches rather than professional plant breeders. This method is cheap, quick and can be used also to transfer CMS to vegetative component. Protoplast fusion can be symmetrical or asymmetrical, but the 4 main stages are the same: isolation of protoplasts of different genotypes, protoplast fusion, protoplast cultivation and plant regeneration (Bhojwani and Dantu 2013).

Principle of the symmetrical fusion is, that nuclear and extra-nuclear content of both components (donor of CMS as well as fertile component we want to transfer to sterile) are fused together. There is a possibility that a fusion occurs also among 2 protoplasts of donor CMS or among 2 protoplasts of fertile component. To ensure that a correct fusion happened (1 protoplast of CMS donor is fused with 1 protoplast of fertile component) the plants must be further grown until they have at least 1 flower. Usage of chemical dyes to visually distinguish the components is also possible (Shankar et al. 2013). With asymmetrical fusion, it is not necessary because cybrids have a combination of 1 protoplast of CMS donor and 1 protoplast of a fertile component. Protoplasts of a CMS donor must be irradiated...
by UV radiation so the nucleus is destroyed (Staxén et al. 1992). Protoplasts of fertile component must be treated with metabolic inhibitors, to remove cytoplasm so just nucleus remains. Crucial is to determine the optimal amount of UV radiation and concentration of metabolic inhibitor for the specific species and genotype.

MATERIAL AND METHODS

Characterization of experimental design and methods

The aim of this research was to determine the optimal UV irradiation needed for Petunia hybrida nucleus destruction. This is a necessary phase of an ongoing research focusing on usage of asymmetrical protoplast fusion in breeding programs of Petunia hybrida. The goal is to use this method to transfer CMS into the fertile component.

In the conducted experiment 3 different genotypes were used, each of them repeated 3 times. The genotypes M–1, M–012, M–033 have back crossed CMS and are used in production of commercial F1 hybrids. This material was provided by Czech biotechnological company Černý-BioPro, Prague.

Protoplast isolation was performed according to the protocol by Meyer et al. 2009 (Meyer et al. 2009) with modified preplasmolytic and degradation enzymatic solution. As a preplasmolytic solution was used 0.6 M mannitol and 0.05 mM MES. The degradation enzymatic solution contained: 1.5% cellulase Onuzaka R10 (Serva), 0.5% Macerozyme R10 (Serva), 0.6 M mannitol, 0.05 M MES, 3 mM CaCl2 and 1 mM KCl.

Leaves were picked in the afternoon from the donor plants grown in a greenhouse, placed into zip lock bag and stored in the fridge with a temperature of 4–6 °C. After 12 hours, leaves were bleached 3 times in commercial bleaching solution SAVO 30 %, epidermis on the bottom side was removed by scalpel. 2 grams of leaves were placed into the Petri dish with 5 ml of preplasmolytic solution, left for 1 hour in darkness, temperature 24 °C. Afterwards the preplasmolytic solution was removed and replaced by degradation enzymatic solution. Petri dish was placed for 2 hours into thermostat with a constant temperature of 26 °C. Every 15 minutes the Petri dish was shaken gently for 3 minutes so the protoplasts can be removed easier from the leaf tissue. Afterwards the solution was filtered through filtration tissue. Protoplasts were rinsed out 3 times in the washing solution in centrifuge (6 min, 740 rpm). The yield and vitality of protoplasts for each genotype were tested.

Before UV irradiation protoplasts were diluted to concentration 100 000 protoplasts/1 ml. 3.5 ml of the protoplast suspension were given into each of the 5 plastic Petri dishes. For the UV irradiation was used UV lamp GV17 with a wavelength 253.7 nm and power 510 μW/cm2. The tested length of the irradiation was 0, 180, 300, 420 and 600 seconds.

Petri dishes with treated protoplasts were placed into the thermostat with a constant temperature of 25 °C. After 10 days, the protoplasts were examined with inversion microscope. The optimal length of UV radiation was, when the nucleus of 100% of protoplasts was destroyed and the cells were not dividing. The same examination was performed also 30 days after UV irradiation.

RESULTS AND DISCUSSION

Irradiated protoplasts were examined using inversion microscope. The aim was to determine what UV irradiation dosage destroys 100 % protoplast nuclei. In table 1 are listed results of 3 repetitions per genotype obtained after 10 days after UV irradiation for duration of 0 sec (Control), 180 sec, 300 sec, 420 sec and 600 sec.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>0 sec</th>
<th>180 sec</th>
<th>300 sec</th>
<th>420 sec</th>
<th>600 sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>M–1</td>
<td>LLL</td>
<td>LLL</td>
<td>DDD</td>
<td>DDD</td>
<td>DDD</td>
</tr>
<tr>
<td>M–021</td>
<td>LLL</td>
<td>LLL</td>
<td>DDD</td>
<td>DDD</td>
<td>DDD</td>
</tr>
<tr>
<td>M–033</td>
<td>LLL</td>
<td>LLL</td>
<td>DDD</td>
<td>DDD</td>
<td>DDD</td>
</tr>
</tbody>
</table>

Legend: L – live protoplasts, D – dead protoplasts; 3 letters per cell represent 3 repetitions for every genotype and UV dosage.
10 days after UV irradiation of a Control (0 sec) the calluses started to form from the fused protoplasts. That means, that the nucleus was not destroyed (Figure 1A). Protoplasts treated with a UV irradiation for 180 seconds (Figure 1B) formed smaller calluses in comparison to Control, cell division occurred. This means that this dosage was not sufficient to destroy the nucleus. Protoplasts treated with UV radiation of 510 μW/cm² for 300 seconds (Figure 1C) started with a cell division, increased their length, but the cell division was not finished, since the formation of nucleus in the newly formed cell did not happen. This means that UV radiation destroyed the protoplast nucleus and this dosage is sufficient and optimal. UV irradiation of 420 seconds (Figure 1D) led to the same results as for 300 seconds. The cell division started, size of protoplasts increased and nucleus of new cell was not formed. 600 seconds of UV irradiation (Figure 1E) led to complete destruction not only of the nucleus but the whole protoplast. Cellular content was dissolved to the cultivation medium.

30 days after UV irradiation were the results like 10 days after irradiation. Calluses of Control and dosage 180 sec were formed. For 300 seconds and more of UV radiation the nucleus did not form in the new cell, which means that this duration was sufficient to destroy all protoplast nuclei.

Results were statistically evaluated with program Statistica 12. It was statistically significant (p = 0.05) that amount of UV irradiation needed for nucleus destruction is not determined by genotype. Due to character of the experiment and binomial distribution of values (1 – live = protoplast nucleus not destroyed by UV irradiation and 0 – dead = protoplast nucleus destroyed, calluses are not forming) sign test was used. On p = 0.05 180 seconds of UV irradiation is not sufficient and nucleus is not destroyed. On p = 0.05 dosage of 300 seconds, 420 seconds and 600 seconds is sufficient but optimal is 300 seconds. The nucleus is destroyed but other cellular content is still vital and able to form callus after asymmetric fusion with a protoplast with nucleus and without cytoplasm.

*Figure 1 Protoplasts 10 days after UV irradiation treatment – genotype M–1*

A) Control (0 seconds)  
B) 180 seconds  
C) 300 seconds  
D) 420 seconds
Symmetric protoplast fusion has a big disadvantage, percentage of correctly fused protoplasts that form callus is very low (Sink 1984). Therefore, asymmetric fusion is more effective way. It is used in breeding for resistance (Bhojwani and Dantu 2013), for interspecific crossing (Guangmin et al. 1998) for higher yield and higher content of proteins etc. In scientific research focusing on Petunias, asymmetric fusion was used to cross incompatible species Petunia and Calibrachoa (Meyer et al. 2009) and for introduction of transformed chloroplasts from Tabaco to Petunia (Sigeno et al. 2009). In horticulture praxis and commercial ornamental plant breeding asymmetric fusion is not used yet (Gerats and Strommer 2009). To destroy the nucleus multiple methods can be used, for example X or Gamma rays, but most frequently is used UV radiation (Lakshmanan et al. 2013). UV radiation is in comparison to the other methods easier to use as Lakshmanan et al. (2013) states. This is the reason why UV radiation was used in the conducted experiment. Crucial is to determine the optimal amount of UV irradiation – voltage and duration. Sigeno et al. (2009) used in the experiment duration of UV irradiation of 20 minutes. This is caused by lower voltage. In our experiment, where 510 μW/cm² was used was optimal UV irradiation from 300 seconds. Irradiation of 10 minutes with 510 μW/cm² caused destruction of not only the nucleus but also of other cellular content, that was dissolved to the medium.

CONCLUSION

Asymmetric fusion brings new opportunities to the flower breeding. This method allows creation of new varieties with unique features that would be not possible to achieve with classical methods due to incompatibility, post fertilization barriers or due to high costs and extensive length of the breeding programme. Cytoplasmic male sterility allows to decrease F1 seed production costs because labour intensive emasculation of maternal component is not needed. Asymmetric protoplast fusion is more effective than back crossing that is used nowadays. Back crossing requires at least 5 or 6 generations to transfer CMS to maternal component and can’t be used for vegetative multiplied component. According to this experiment the optimal amount of UV irradiation to destroy Petunia hybrida nucleus is 510 μW/cm² for a duration of 300 seconds. Longer irradiation tends to destroy other cellular content as well, lower dosage does not destroy the nucleus.

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REFERENCES


