Investigation of the *in vitro* regeneration of mericlones in the caribe variety of carnation

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Summary: *In vitro* culture conditions were experimented for the relatively sensitive, but very esthaetic "Caribe" variety of carnation with uniformly dark violet flowers. Regeneration of new plants from shoot apex meristems can be significantly improved by the combined addition of very low amounts of indolebutiric acid, benzyladenine and gibberelic acid, dissolved in the Murashige-Skoog nutrient medium. Callus formation,as a prerequisite for the induction of somaclonal variability, can be achieved successfully with certain molar ratios between 2,4-dichlorophenoxyacetic acid and benzyladenine. Acclimation of the obtained mericlones to the *ex vitro* conditions was also evaluated.

Introduction

Carnation is one of the most widespread ornamental plants and the high number of similar individuals is mainly obtained by *in vitro* mericlone micropropagation. This method also enables the cultivation of healthy plantlets lacking any kind of infection. The main inconvenience of this multiplication is that the young plants obtained in artificial media are very sensitive to the different environmental stress conditions that normally occur in natural habitats. Disinfection, genetic control, developmental regulation and acclimation have to be achieved in a cumulative manner and this makes very difficult the industrial scale production of the desired plant material. This is the reason why at present many attempts are done in order to improve the efficiency of micropropagation of useful plant species [1, 2, 8].

The developmental control of mericlones is largely based on the interactions between several growth regulator substances and it is possible to influence this interaction by addition of synthetic or natural phytohormones externally. Auxin- and cytokinin-type regulators can usually stimulate histogenetic and organogenetic processes and sometimes gibberelins also enhance the action of the formerly mentioned two categories of plant hormones.

The aim of this work is to present some possibilities to improve the mericlone regeneration, micropropagation and organogenetic potential of a rare variety of carnation with dark violet flowers.

Material and method

Among the several varieties of carnation (*Dianthus caryophyllus* L.) there is one with very high quantities of betalains that accumulate in the vacuolar sap of petal cells, so the flower becomes uniformly dark violet. This "Caribe" variety is more sensitive to pathogenic infection and other environmental stresses that most of the other cultivated carnation types. It has a thinner stem with less developed sclerenchymatic tissues and a slightly smaller flower. Its high-scale reproduction is more difficult, but an ever-increasing demand has been registered for this delicate and attractive carnation variety [5]. This demand can be satisfied by more efficient micropropagation and acclimation procedures, which suppose a better knowledge of the hormonal regulation of its morphogenetic processes and the interacting internal and external factors that determine its growth rate and vitality.

Culture conditions and media

The *in vitro* cultures were started from shoot apical meristems with a diameter of about 2 mm, isolated under axenic conditions and inoculated on sterile Murashige-Skoog (MS) nutrient medium gelified with agar-agar [4, 7]. The control was grown on pure MS medium, while the other experimental variants contained the following developmental stimulators:

a) 4 µM benzyladenine (BA), i.e. a synthetic cytokinin

b) 0.5 µM naphtylacetic acid (NAA, a synthetic auxin), 0.5 µM kinetin (K, a cytokinin) and 100 mg l^{-1} kazein (Kaz) as an organic nitrogen source, added to a MS medium without B-type vitamins, inositol and glycin

c) 2 µM gibberelic acid (GA_3), 3 µM indolebutiric acid (IBA, a synthetic auxin) and 5 µM BA.

For the induction of callus formation in the *in vitro* cultures, the MS medium was supplemented with the following amounts of 2,4-dichlorophenoxiacetic acid (2,4–D as a synthetic auxin also used as a herbicide) and benzyladenine:

1) 0.5 mg l^{-1} 2,4–D + 2.0 mg l^{-1} BA
2) 1.0 mg l^{-1} 2,4–D + 1.5 mg l^{-1} BA
3) 1.5 mg l^{-1} 2,4–D + 1.0 mg l^{-1} BA
4) 2.0 mg l^{-1} 2,4–D + 0.5 mg l^{-1} BA

The *in vitro* cultures were kept for 25 days in a growth chamber at 22 °C with a daily photoperiod of 16 hours illumination (with a light energy flux of 30 W m^{-2}) and 8 hours darkness. Shoot length, rhyzogenetic capacity, number of internodes and callus formation were evaluated biometrically and statistically processed. Acclimation of the *in vitro* regenerated plants to *ex vitro* conditions was performed in large pots with vermiculite as a solid substrate in a greenhouse that was opened for longer and longer daily periods, in order to achieve a progressive habituation with dry air [6, 7].

Results and discussion

The shoot growth of the mericlones regenerated from the apical meristem was almost doubled compared to the control on the culture medium enriched with a combination of gibberelin (GA_3), auxin (IBA) and cytokinin (BA). This supplementation with micromolar amounts of external growth stimulators, interacting with the endogenous hormones produced by the plant cells, resulted in a very significant enhancement of cell divisions and elongation processes. The benzyladenine together with the cytokinin produced in the meristematic cells, stimulated the rate of mitotic process, while the interaction of auxins and gibberelic acid led to an enhanced cell elongation in the internodes of the stem and in the developing young leaves. This means that under these conditions the new plantlets grow much faster, they reach maturity and produce flowers in a much shorter period of time. Addition to the MS medium of benzyladenine alone results in a moderate stimulation of shoot growth, while the development of the regenerated mericlones is inhibited by around 50% in the absence of B-type vitamins, even if the nutrient medium was supplemented with exogenic auxin (NAA), citokynin (K) and kazein *(Figure 1)*.

Gallus formation occured in different degrees on the culture media. It was most intense on the MS medium

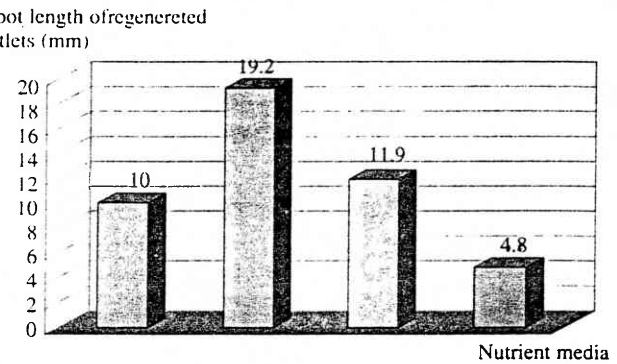

Figure 1 The influence of different growth factors on shoot length of in vitro cultured carnation mericlones

lacking vitamins and enriched with NAA, K and kazein. Cytokinins usually stimulate callus induction because they stimulate cell divisions that lead to the formation of an undifferentiated cell mass. But the addition of 4 µM benzyladenine did not enhance significantly the callus formation, because it did not lead to an improved hormonal balance in the *in vitro* cultured meristematic cells. As compared to the control, the combination of GA_3, IBA and BA intensified the generation of callus, but not as much as the joint addition of NAA, kinetin and kazein did *(Figure 2)*.

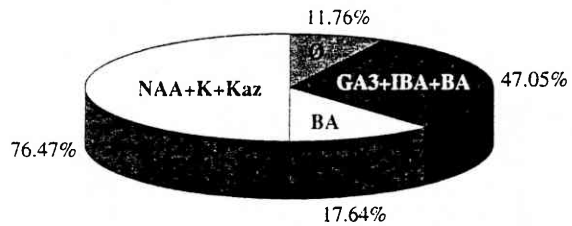

Figure 2 Influence of different combinations of growth regulator substances on the callus formation in shoot meristem cultures of the "Caribe" variety of carnation

It has to be mentioned that callus formation is a positive phenomenon if one intends to initiate new individual varieties by means of somaclonal variability, but it is an undesirable side-event if the goal is to obtain more descendants that have to be identical with the formerly selected mother plant [1, 3, 9].

Root formation was not stimulated by either of the experimental setups and it was mostly inhibited by addition of 4 µM benzyladenine as an extra amount of cytokinin. This results can be explained by the fact that the ratio between total auxin and cytokinin concentrations in the cells was modified in favor of the cytokinins, which stimulate caulogenetic regeneration, while rhyzogenesis needs a higher auxin: cytokinin ratio [8]. The slightest inhibition of root formation was observed in the medium supplemented with the combination of 2 µM GA_3, 3 µM IBA and 5 µM BA *(Figure 3)*.

The results show that the different organogenetic processes of the same plant require different hormonal conditions, a fact that makes practically impossible the

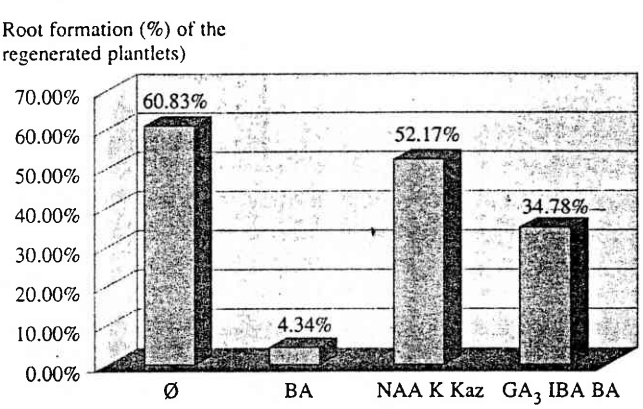

Figure 3 Influence of growth factors on the rhyzogenesis of in vitro regenerated plantlets of the "Caribe" variety of carnation

identification of an optimal medium for all of the developmental processes involved in the regeneration of entire plant organisms.

Induction of callus formation in cultures of the regenerated shoots as a prerequisite for somaclonal variability and selection of new carnation types was assayed with the combined addition of different amounts of 2,4–D and BA. Callus formation was mostly stimulated by 1.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA, and it was less enhanced by 0.5 mg l^{-1} 2,4-D and 2.0 mg l^{-1} BA. It is very difficult to comment these results because the concentrations of endogenous auxin and cytokinin are not known, but under the described conditions it seems that slightly increased ratios between the externally added 2,4–D and BA exhibit the most favorable influence on callus formation of the basal segment of stems *(Figure 4)*.

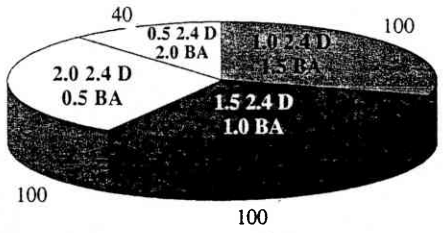

Figure 4 Influence of different auxin: cytokinin ratios on callus formation of carnation stem segments

A very high percentage (96.8%) of the *in vitro* obtained mericlones belonging to the "Caribe" variety of carnation successfully survived to the progressive acclimation to *ex vitro* conditions that imply lower air humidity, less controlled nutritional conditions in the soil and different variations of photon flux density. This is a very good result concerning the success in reaching maturity and flowering. Still an even more efficient method of cultivation has to be experimented for a more profitable large-scale micropropagation.

Conclusions

The *in vitro* micropropagation of the relatively sensitive but very attractive "Caribe" variety of carnation can be improved by the combined addition of micromolar concentrations of growth regulators to the nutrient medium.

Shoot elongation of the regenerated, germ-free mericlones can be significantly stimulated with 2 µM gibberelic acid, 3 µM indolebutiric acid and 5 µM benzyladenine.

Instead of organogenesis, callus formation is enhanced by naphtylacetic acid, kinetin and kazein added to a MS medium without vitamins.

Root formation is mainly sustained by NAA and kinetin and it is mostly inhibited by 4 µM benzyladenine without being combined with exogenous auxins.

The callus formation needed for the induction of somaclonal variability is improved by moderately increased 2,4–D : BA ratios.

The *in vitro* micropropagated mericlones can be successfully acclimatized to the greenhouse conditions in order to obtain a high number of healthy carnation plants with dark violet flowers.

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