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THE COURSE AND EFFICIENCY OF ORGANOGENESIS ON LEAF EXPLANTS OF PLUM 'WĘGIERKA ZWYKŁA' (*PRUNUS DOMESTICA* L.) INDUCED BY CYTOKININS.

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ABSTRACT

The effects of organogenesis on *in vitro* leaf explants of plum ‘Węgierka Zwykła’ cultured on media with either TDZ or BAP were compared. TDZ was applied at concentration of 0.5 – 1.65 mg/l in combination with 2,4 – D, and BAP at concentration of 3.0 or 5.0 mg/l with one of the auxins: IBA, NAA or 2,4 – D. As the result two different ways of organogenesis were observed: the efficient (60%-72%) indirect organogenesis induced by TDZ, and direct organogenesis, or with only very small callus formation on media supplied with BAP. Explants cultured on media with TDZ produced big masses of callus followed by differentiation of numerous adventitious buds in clusters while on media containing BAP only single buds of smaller size were formed.

Key words: BAP, cytokinin, organogenesis, plum, *Prunus domestica* L., TDZ

Abbreviations:

- BAP - 6 benzylaminopurine
- 2,4-D - 2,4-dichlorophenoxyacetic acid
- IBA - 3- indolylbutyric acid
- MS - Murashige and Skoog medium
- NAA - 1-naphtaleneacetic acid
- TDZ - thidiazuron [1-phenyl-3-(1,2,3-thiadiazol-5-yl)]urea

INTRODUCTION

It is difficult to overestimate the meaning of plant tissue cultures in contemporary breeding methods. Different *in vitro* techniques are applied according to the expected results of laboratory procedures. In some cases it is important to obtain clones similar to the mother plant, while in the other – it is desired to induce somaclonal variation in the cultivated tissue for development of new traits. In general it is relatively easy to induce variation during the course of regeneration of explants *in vitro* but the tissue most prone to spontaneous genetic alterations in such conditions is callus. The regeneration through the callus stage is therefore desired when we want to stimulate the genetic variability of the cultivated material, otherwise direct regeneration methods, omitting the phase of callus formation, should be advised.

The regeneration processes in explants cultivated *in vitro* are under control of a number of both the internal factors, determined by the explant itself (genotype and physiological status of maternal plant, type of explant used), and the external ones resulting from the conditions of the cultivation of the tissues. Among them most important are the kind of plant regulators introduced into culture medium, their concentrations and proportion between auxins and cytokinins. Cytokinins that stimulate cell divisions, and differentiation of adventitious buds, are crucial for morphogenesis *in vitro*. For most fruit trees high cytokinin/auxin ratio favours caulogenesis in cultivated tissues [5].

So far, for leaf explants from different cultivars of *Prunus domestica*, the application of high doses of cytokinin along with lower amounts of auxins was necessary to obtain organogenesis, and the most efficient in that respect appeared to be BAP, and TDZ. The high efficiency of BAP was described for regeneration in explants of ‘Bluefre’ [2], whereas TDZ stimulated considerably caulogenesis in cultivars ‘Damas de Toulouse’, ‘Węgierka Zwykła’, ‘Węgierka Łowicka’, ‘Węgierka Dąbrowicka’, ‘Čačanska Rodna’, ‘Čačanska Najbolia’ and ‘Stanley’ [3, 7, 11, 12]. In all those cases only indirect organogenesis was observed. However Yancheva [1994] described also direct organogenesis induced in explants of ‘Stanley’ and ‘Kjustendilska sliva’ on media supplemented with TDZ.

In the present paper results of application of two cytokinin – TDZ or BAP for regeneration *in vitro* from leaf explants of plum ‘Węgierka Zwykła’ are compared.

MATERIALS AND METHODS

The source of leaf explants was a stabilised *in vitro* shoot culture of ‘Węgierka Zwykła’ that had been cultivated on proliferation MS medium [10] with the addition of 0.5 mg/l BAP and 0.1 mg/l of IBA [8], solidified with 5 g/l agargel (Sigma) at pH 5.5 adjusted with NaOH.

The first 2-4 fully developed leaves with petiole were sampled for regeneration experiments 2-3 weeks after subculture. The leaves, cut across the midrib, with the distal part removed, were placed abaxial side on the agar in 100 ml Erlenmeyer flasks containing 25 ml of medium, five explants in each. The flasks were kept in the dark for the first three weeks. After that period the explants were transferred onto the fresh medium of the same composition, the flasks being exposed to the 16/8 light/dark cycle of about 70 µmol $m.^{-2}$ s^{-1} irradiance during next three weeks, at temperature 23±2°C. At the end of regeneration procedure the appropriate observations and measurements were carried out.

The composition of medium was based on modified MS nutrients with some chemicals reduced by half (NH_4NO_3, KNO_3, $CaCl_2$ x 2 H_2O, KJ, $CoCl_2$ x 7 H_2O), and supplied with 1.0 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 100 mg/l myoinositol, 100 mg/l casein hydrolysate, 2.0 mg/l glycine, 10 mg/l ascorbic acid, 10 mg/l citric acid and sucrose 30 g/l. The growth regulators were applied prior to autoclaving. BAP was used in combination with three auxins, and three concentrations of TDZ were combined with two different concentrations of 2,4 – D (Table 1).

The following characteristics were described at the end of experiment: the percentage of regenerating explants, mean number of buds per explants, size of explants (at the groups 1.5cm ≤, 1.0 cm, 0.5 cm and M = for buds smaller than 0.5 cm), and fresh weight of explants (explants with developed callus and/or adventitious buds).

The experiment was conducted in 12 replications, each consisting of one flask with five explants. The results were statistically verified using ANOVA and Student's – t test at p = 0.05. ANOVA for experiments with unequal number of replications was applied for evaluation of some of parameters (mean number of buds per explant, mean size of buds beyond the M class, contribution of M-buds in total number of buds).

RESULTS

The regeneration processes were observed on explants cultivated on each of the tested media although their effectiveness varied significantly depending on the cytokinin used (Table 1).

Table 1. The efficiency of regeneration and the size of differentiated buds on media supplemented with two cytokinins: TDZ or BAP after 6 weeks of cultivation. The numbers represent results from 12 replications.

Cytokinin [mg/l]	Auxin [mg/l]	Regeneration [%]	Number of buds per explant [x]	Contribution of buds from group M in total number of buds [x] [%]	Size of buds beyond the group M [x] [mm]	Fresh weight of explants [mg]
3.0 BAP	0.2 IBA	10.0 bc	2.8 c	93.3 ab	50.0 bcd	144.9 c
3.0 BAP	0.2 NAA	1.7 c	3.0 c	100.0 a	-	61.8 c
3.0 BAP	0.2 2,4 - D	6.7 bc	2.3 c	72.3 bc	50.0 bcd	76.2 c
3.0 BAP	0.5 IBA	25.0 b	2.8 c	65.7 bc	51.2 bcd	109.9 c
5.0 BAP	0.5 IBA	13.3 bc	4.1 c	90.9 ab	50.0 bcd	76.2 c
1.65 TDZ	0.2 2,4 - D	71.7 a	11.7 a	77.6 abc	67.7 abc	494.0 ab
1.0 TDZ	0.2 2,4 - D	66.7 a	10.3 ab	65.1 bc	70.7 abc	624.3 a
0.5 TDZ	0.2 2,4 - D	63.3 a	7.4 b	55.6 c	77.1 a	399.2 b
1.65 TDZ	0.1 2,4 - D	60.0 a	9.7 ab	72.9 bc	78.9 a	456.6 b
1.0 TDZ	0.1 2,4 - D	65.0 a	8.7 ab	57.1 c	72.1 a	440.5 b
0.5 TDZ	0.1 2,4 - D	60.0 a	9.5 ab	73.1 abc	71.5 ab	318.3 b

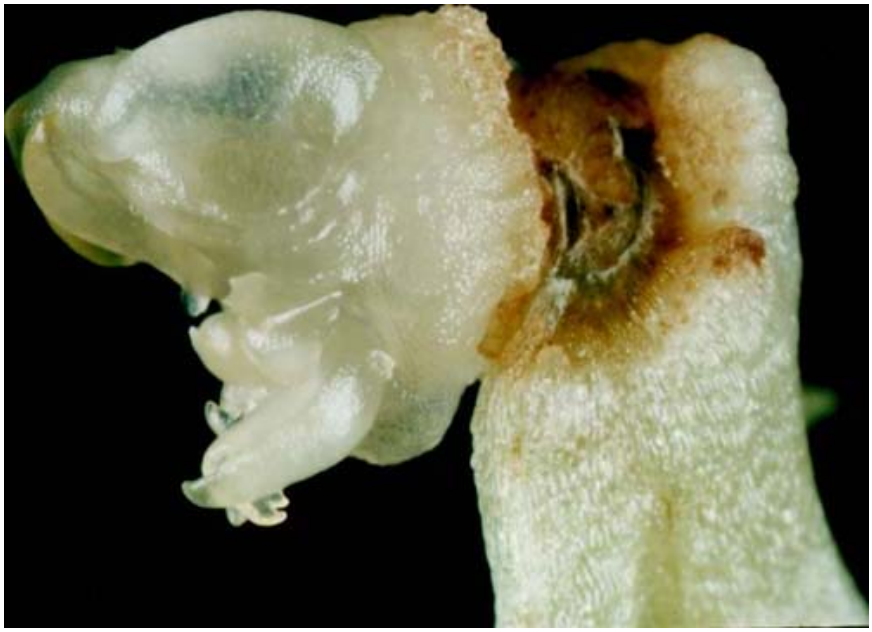
Means followed by same letter (a, b, c) do not significantly differ at p = 0.05.
[x] Replications, in which the buds did not develop, were not taken into account in calculations. Statistical analysis for given parameters were carried out in version for unequal number of replications

Application of TDZ induced organogenesis on more than half of excised explants (60 – 72%) regardless of the concentration used. The developing, adventitious buds were numerous (ca. 9-12 per explant) and although the contribution of small buds – M – was here significant, the presence of relatively big ones (68-78 mm) was noted as well. In all cases the development of buds was preceded by the formation of a compact callus of big size, that contributed to a great part of explant fresh weight (ca. 320 – 624 mg). The regeneration processes in all those cases took the form of indirect organogenesis (Phot 1). In comparison to TDZ the effectiveness of BAP at concentrations used was relatively small (ca. 2-25%), no matter which kind of auxin was used (Phot. 2 and 3). The numbers of adventitious buds formed was insignificant (2-4), and they were frequently single, usually belonging to the category of small ones (M) or only slightly bigger. Only very small traces of differentiated callus were visible, and this points on direct organogenesis, especially in case of medium supplemented with 3.0 mg/l BAP and 0.2 mg/l IBA (Phot. 2). This fact was proved by microscopic examination which will be the subject of a separate publication.

Phot. 1. The explants of 'Węgierka Zwykła' after 6 weeks of culture on medium supplemented with 1.65 mg/l TDZ and 0.2 mg/l 2,4 – D.

Phot. 2. The explants of 'Węgierka Zwykła' after 6 weeks of culture on medium supplemented with 3.0 mg/l BAP and 0.2 mg/l IBA.

Phot. 3. The regenerating adventitious bud on explant of 'Węgierka Zwykła' 21 days after the onset of regeneration on medium supplemented with 3.0 mg/l BAP and 0.5 mg/l IBA.



DISCUSSION

Using two different cytokinins the present authors succeeded to induce two different ways of regeneration from *in vitro* leaf explants of plum 'Węgierka Zwykła': indirect organogenesis occurring with high frequency when TDZ was introduced into the medium, and organogenesis with small differentiated callus or even direct one – occurring without preceding callus stage, however with low effectiveness – when BAP was applied. This cytokinin was thus far reported as being a high efficient regeneration factor in *in vitro* cultures of plum leaf explants only in case of 'Bluefre' (up to 100% of regenerated explants) [2].

The crucial point for success in this case could have been the cultivation of respective explants in red light during two consecutive subcultures, while in most protocols described for the genus *Prunus* the beginning of morphogenetic processes (induction) took place in darkness followed by the cultivation in changing light and dark periods [3, 7, 11, 12]. Light in *in vitro* cultures not only provides energy for photosynthesis, but possesses also considerable potential for regulation of many metabolic pathways. In consequence the processes of cytodifferentiation and morphogenesis are under control of light condition [1].

The new artificial cytokinin, TDZ, was for the first time used for cultivation of leaf explants of the genus *Prunus* by Escalletes and Dosba [1993], but in those experiments the results of regeneration were not very satisfactory. In following experiments the application of TDZ gave much better effects. Using the similar protocol for regeneration Nowak and Miczyński [1997] were able to obtain formation of adventitious buds of 'Čačanska Rodna', 'Stanley' and 'Čačanska Najbolia' with higher efficiency than in previous research (70%, 50% and 40% respectively). Also organogenesis at group of 'Węgierka' cultivars was reported to occur at a comparatively high frequency both - in earlier investigations of Lis [1995] for 'Węgierka Dąbrowicka', Węgierka Łowicka', 'Węgierka Zwykła' (81%, 10%, 31%), or in experiments of Nowak and Miczyński [1996] with ca. 50% of regenerated explants, and finally in the present authors work. One should emphasise the fact that results presented here for the examined range of concentrations for combined action of both TDZ and 2,4 – D are very uniform.

TDZ has a strong cytokinin activity, and this was probably the reason why its application for regeneration of 'Węgierka Zwykła' in tissue cultures, resulted in our experiments in formation of numerous buds which seldom developed into shoots without subculture onto proliferating medium. Similar manner of differentiation of bud clusters which did not grow into shoots was already described by other explorers for media supplemented with TDZ [4, 6].

The only known case of direct organogenesis reported for regeneration in vitro of plum cultivars, was described for 'Stanley' and 'Kjustendilska sliva' [13]. It was induced by the combination of TDZ/IBA according to the

protocol which the present authors applied previously for ‘Stanley’ [12] with different effect – of indirect organogenesis. This fact points out, for some others, still unknown factors, which can have essential meaning for the differentiation of plant tissue *in vitro*.

For many purposes of genetic engineering efficient methods ensuring least genetic variability resulting from conditions of tissue regeneration *in vitro* would be the most desired. In such cases direct methods of regeneration should be applied. And though there are reports about high genetic stability of plants regenerated from callus of *Prunus amygdalus* [9], it should be expected, that the use of 2,4 – D for differentiation, will enhance variability. On the other hand it could be preferable for some breeding purposes to stimulate this kind of variability during the regeneration process. Therefore the choice of regeneration technique should depend on the purpose for which the regenerated plants are obtained.

CONCLUSIONS

1. Modified medium MS with vitamins, antioxidants and supplemented with auxins and either TDZ or BAP is suitable for adventitious bud regeneration on in vitro leaf explants of plum 'Węgierka Zwykła'.
2. Cytokinin TDZ introduced into medium induced indirect organogenesis with high frequency, numerous buds being formed on one explant.
3. The effectiveness of organogenesis on media supplemented with BAP was relatively small in comparison to TDZ, and only small, or not at all, differentiated callus preceded regeneration.

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