FACTORS MODIFYING REGENERATION *IN VITRO* OF ADVENTITIOUS SHOOTS IN FIVE RED RASPBERRY CULTIVARS

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A B S T R A C T

A number of experiments were undertaken, using the leaves from four-week-old donor shoots of five raspberry cultivars, in an attempt to improve the process of regeneration of adventitious shoots *in vitro.* The cultivars used were 'Beskid', 'Canby', 'Malling Seedling', 'Norna' and 'Veten'. The medium for donor shoots of 'Malling Seedling' and 'Canby' should contain N_6 mineral salts (Chu et al., 1975) and for the 'Beskid', 'Norna' and 'Veten', Murashige and Skoog's (1962) mineral salts, 0.6 mg l^{-1} 6-benzylaminopurine and 0.1 mg l^{-1} indole-3-butyric acid. Murashige and Skoog's medium supplemented with thidiazuron and indole-3-butyric acid (both at 0.1 mg l^{-1}) and with FeEDTA replaced with FeEDDHA is recommended for regeneration. The medium pH for the regeneration of 'Canby' and 'Malling Seedling' should be lowered to 4.6. The highest regeneration potential was observed in 'Beskid', and the lowest in 'Malling Seedling'.

Key words: auxin IBA, FeEDDHA chelate, mineral complex, pH, thidiazuron

INTRODUCTION

Biotechnological methods are valuable in the breeding of new raspberry cultivars that are better adapted to local soil and climate conditions. *In vitro* transformation and selection of desired genotypes is a particularly useful method. In order to achieve optimal results from biotechnological methods, it is necessary to know how to regenerate whole plants from single cells, either by adventitious shoots or by somatic embryogenesis.

In raspberries, the ability to produce adventitious shoots varies from genotype to genotype (Cousineau

and Donnelly, 1991; Turk et al., 1994; Graham et al., 1997). Therefore, it is often necessary to design a protocol of regeneration of a particular cultivar on the base of own experiments using existing knowledge as guiding instructions.

The purpose of this study was to determine the optimum composition of medium on which donor shoots are grown in order to increase their competence for regeneration and medium for regenerating adventitious shoots from leaves of five raspberry cultivars.

MATERIAL AND METHODS

The following raspberry cultivars were used in this study: 'Beskid', 'Canby', 'Malling Seedling', 'Norna' and 'Veten'. *In vitro* grown shoots were used as leaf donors for experiments on adventitious shoot regeneration. Leaf explants were taken from virus-free shoot cultures, which had been propagated *in vitro* for three years on Murashige and Skoog's (1962) medium, modified by increasing FeEDTA concentration to 53.3 mg l^{-1} and $MgSO_4$ x 7 H_2O to 525 mg l^{-1} and supplemented with: vitamins according to Lloyd and McCown (1981), 3.6 μM (0.8 mg l^{-1}) of 6-benzylamino purine (BAP), 0.5 μM (0.1 mg l^{-1}) of indole-3-butyric acid (IBA), 30 g l^{-1} of sucrose, and solidified with 7 g l^{-1} of bioMerrieux agar. The pH of the medium was adjusted to 5.6 before being autoclaved. The cultures were subcultured every six weeks onto fresh shoot propagation medium.

The youngest, fully developed leaves, taken from four-week-old donor cultures, were used for regeneration. The leaves were cut across their midribs with a scalpel and placed on the medium so that their undersides were in contact with it. Several experiments were then performed in order to determine which factors promote adventitious regeneration.

Experiments on composition of the medium on which donor cultures were grown

The basal medium on which donor shoots were grown was the same as for shoots propagation with the only exception that BAP concentration was lowered to 0.6 mg l^{-1}. The following were assessed:

- The effect of cytokinins – 0.1 or 0.2 mg l^{-1} TDZ and 0.8 mg l^{-1} BAP at the constant level of 0.1 mg l^{-1} naphthalene-1-acetic acid (NAA).
- The effect of combinations of growth regulators, using (in mg l^{-1}): 1.0 BAP + 3.0 kinetin (KIN); 3.0 KIN; 0.5 BAP + 0.1 gibberellic acid (GA_3); 5.0 6-γ-γ-(dimethyl-allylamino)-purine (2iP); 1.0 BAP + 3.0 2iP, all of these at the constant level of 0.1 mg l^{-1} NAA, and the medium without growth regulators.
- The effect of mineral composition of the medium, using: MS, Q-L (Quoirin and Lepoivre, 1977), N_6 (Chu et al., 1975) and B5 (Gamborg et al., 1968) mineral components.

Experiments on composition of the medium on which leaves were stimulated for regeneration

The basal regeneration medium consisted of complete MS salts (Duchefa No. 221), Lloyd and McCown (1981) vitamins, 30 g l^{-1} sucrose, 8 g l^{-1} Bacto agar, pH 5.6. The folowing were assessed:

- The effect of 0.1, 0.5 and 1.0 mg l^{-1} TDZ and 0.05 and 0.1 mg l^{-1} IBA in all combinations as well as the effect of 0.05 and 0.1 mg l^{-1} TDZ together with 0.1 mg l^{-1} IBA;
- The effect of a kind of auxin, using: 0.1 mg l^{-1} IBA, 0.2 mg l^{-1} indole-3-acetic acid (IAA) and 0.2 mg l^{-1} NAA together with 0.1 mg l^{-1} TDZ;
- The effect of mineral composition of the medium, using M-S, Q-L, N_6 and B5 mineral components;
- The effect of iron sources, using 60 mg l^{-1} FeEDTA; 40 mg l^{-1} FeEDTA + 50 mg l^{-1} FeEDDHA and 50 mg l^{-1} FeEDDHA, each at three pH values – 4.6, 5.0 and 5.6.

All cultures were maintained at a constant temperature of 23°C. The propagation cultures and donor shoots were maintained under fluorescent light at irradiation between 50 and 60 µmol m^{-2} sec^{-1}. The regeneration cultures were incubated for seven days in darkness and then maintained under fluorescent light at irradiation 20 µmol m^{-2} sec^{-1}.

The regeneration results were evaluated after six weeks and the following data were recorded: the number of responding leaves, the percentage of necrotic leaves and the number of adventitious shoots per responding leaf.

Each treatment was applied to at least 25 explants using five Petri dishes, each containing five leaves. The data, recorded in percentages, were transformed using the Bliss function. Data for the number of responding leaves were transformed using the Freeman-Tukey function. All data were statistically elaborated using analysis of variance, followed by means separation using Duncan's multiple-range t-test at P=0.05.

RESULTS

The effect of TDZ in the donor shoots and regeneration media

There were no significant differences in the number of responding leaves obtained from shoots cultured on media containing TDZ or BAP. In 'Beskid', all of the leaves responded, regardless of which medium was used. In 'Canby', 'Malling Seedling' and 'Norna', the proportion of responding leaves was highest when they were obtained from shoots grown on medium containing BAP but in 'Veten', when they were obtained from medium containing 0.1 mg l^{-1} TDZ. In 'Malling Seedling' and 'Norna', regeneration of leaves obtained from shoots grown on the medium containing 0.2 mg l^{-1} TDZ was not possible due to the hyper-hydration (data not shown).

Direct regeneration of adventitious shoots, in all three cultivars, was most efficient when the regeneration

medium contained 0.1 mg l^{-1} TDZ. At higher concentrations, TDZ caused leaf blackening, or induced hyperhydration usually followed with necrosis of the adventitious shoots.

On the regeneration medium containing 0.05 mg l^{-1} TDZ, the proportion of responding leaves was lower, although the difference was not statistically significant (Tab. 1).

The effect of auxins in the regeneration medium

IBA, at concentrations of 0.05 mg l^{-1} and 0.1 mg l^{-1}, did not affect regeneration as unequivocally as did TDZ. In 'Veten', regeneration was more efficient on medium containing 0.05 mg l^{-1} IBA but in 'Malling Seedling', regeneration was better on the medium containing 0.1 mg l^{-1} IBA (Tab. 1). In 'Malling Seedling' and 'Norna', the proportion of necrotic leaves was higher on medium containing 0.1 mg l^{-1} TDZ in combination with 0.05 mg l^{-1} IBA.

Of the three auxins tested, IBA effectively induced regeneration in all of the cultivars. IAA gave poor results with 'Norna' but was particularly effective in 'Canby'. NAA gave poor results with all of the cultivars tested (Tab. 2).

The effect of growth regulators in the donor shoot medium

The regeneration rate from leaves obtained from shoots grown on the media containing BAP + KIN and BAP + GA_3 was high in all of the cultivars tested. From leaves obtained on shoots propagated on medium containing KIN alone, the regeneration rate was high only in 'Beskid' and 'Veten'. The regeneration rate from leaves obtained on shoots cultured on the medium without growth regulators was low in all of the cultivars tested (Tab. 3).

The effect of mineral composition in the donor shoots and regeneration media

The mineral composition used in the donor culture medium did not have any significant effect on the number of responding leaves, except in 'Malling Seedling', where the number of responding leaves was significantly higher with the use of N_6 and B5 salts. B5 medium decreased the number of adventitious shoots of 'Beskid' cultivar. In the 'Canby' and 'Malling Seedling' cultivars, the number of shoots was the highest with the use of N_6 salts. The mineral composition of donor shoot medium did not have influence on the number of adventitious shoots of 'Norna' and 'Veten' cvs. (Tab. 4).

The mineral salt compositions in the regeneration medium did not influence the number of regenerating leaves of 'Beskid' and 'Malling Seedling' nor adventitious shoots of 'Malling Seedling' and 'Veten' cultivars. In 'Canby' cultivar, Q-L and B5 significantly lowered both parameters of regeneration. The worst results of regeneration in all cultivars were observed with B5 salts (Tab. 5).

T a b l e 1 . The dependence of the regeneration of adventitious shoots of raspberry on the concentration of TDZ and IBA in the regeneration medium

Growth regulators [mg l^{-1}]		% of responding leaves			% of necrotic leaves			No. of adventitious shoots per responding leaf		
TDZ	IBA	Malling Seedling	Norna	Veten	Malling Seedling	Norna	Veten	Malling Seedling	Norna	Veten
0.1	0.05	17c*	44b	82d	84b	31b	2a	1.0b	1.6a	3.2b
	0.1	41d	44b	49bc	33a	9a	22b	2.2d	1.8a	3.1b
0.5	0.05	14c	11a	67cd	96c	64c	55c	1.7c	1.8a	2.3a
	0.1	10bc	19a	15a	90bc	63c	98c	1.0b	1.3a	1.6a
1.0	0.05	0a	9a	43b	100d	80cd	70cd	0a	2.5b	1.8a
	0.1	5b	14a	15a	100d	86d	88de	1.0b	1.7a	1.8a
0.1		29c	44b	67b	60a	19a	10a	1.6c	1.7ab	3.2b
0.5		12b	15a	39a	93b	64b	82b	1.3b	1.5a	2.0a
1.0		1a	15a	27a	100c	83c	80b	0.5a	2.1b	1.8a
	0.05	7a	20a	64b	96b	59a	37a	0.9a	2.0a	2.4a
	0.1	16b	25a	24a	83b	52a	74b	1.4b	1.6a	2.2a

*Means followed by the same letter do not differ at P=0.05

T a b l e 2 . The influence of auxins in the regeneration medium on the adventitious shoot regeneration in raspberry

Auxin [mg l^{-1}]	No. of responding leaves (n = 5)				No. of shoots per responding leaf			
	Beskid	Canby	Norna	Veten	Beskid	Canby	Norna	Veten
IBA 0.1	3.6b*	3.8b	2.2b	4.8b	2.3b	4.4b	1.1a	4.4c
IAA 0.2	3.6b	4.6c	0.4a	3.8b	1.7ab	4.2b	0.6a	2.8b
NAA 0.2	1.0a	1.2a	0.4a	1.8a	0.9a	0.7a	0.4a	0.7a

*Explanations, see Table 1

T a b l e 3 . The influence of growth regulators in the donor shoots medium on the adventitious shoot regeneration in raspberry

Growth regulators [mg l^{-1}]	No. of responding leaves (n = 5)					No. of shoots per responding leaf				
	Beskid	Canby	Malling Seed.	Norna	Veten	Beskid	Canby	Malling Seed.	Norna	Veten
BAP 1+KIN 3	5.0b*	4.0b	2.0b	2.0a	4.8a	5.7b	2.5a	1.0a	1.5ab	3.3a
BAP 0.5+GA_3 0.1	5.0b	5.0c	2.6b	2.4a	4.4a	5.6b	2.6a	1.6a	2.2b	5.4b
KIN 3	4.8b	1.6a	0.6a	0.8a	4.8a	3.9a	1.9a	0.7a	0.8a	5.2b
None	4.0a	0.8a	0.6a	0.8a	4.4a	4.1a	1.2a	1.2a	0.9a	2.7a

*Explanations, see Table 1

Table 4. The influence of the mineral composition of the medium for donor shoots on the adventitious shoot regeneration in raspberry

Mineral complex	No. of responding leaves (n = 5)					No. of shoots per responding leaf				
	Beskid	Canby	Malling Seed.	Norna	Veten	Beskid	Canby	Malling Seed.	Norna	Veten
MS	5.0a*	4.8a	2.6a	3.0a	4.6a	5.4b	2.5a	1.2a	1.4a	4.4a
Q-L	5.0a	4.8a	2.6a	2.0a	4.6a	5.0b	2.4a	1.4a	1.6a	4.6a
N_6	4.8a	5.0a	4.2b	3.4a	5.0a	4.2ab	5.8b	2.4b	1.9a	3.7a
B5	4.8a	4.8a	3.8b	3.2a	5.0a	3.1a	3.0a	1.5a	1.7a	4.2a

*Explanations, see Table 1

Table 5. The influence of the mineral composition of the regeneration medium on the adventitious shoot regeneration in raspberry

Mineral complex	No. of responding leaves (n = 5)					No. of shoots per responding leaf				
	Beskid	Canby	Malling Seed.	Norna	Veten	Beskid	Canby	Malling Seed.	Norna	Veten
MS	3.7a*	3.0b	1.0a	1.6b	4.6b	2.9b	2.2b	0.5a	1.6b	4.4a
Q-L	3.2a	1.8ab	0.8a	0.8ab	4.4b	1.0a	1.0ab	0.7a	0.8ab	4.6a
N_6	3.2a	3.2b	0.4a	1.0ab	4.2b	3.2b	1.8b	0.4a	1.0ab	3.7a
B5	1.6a	0.6a	0.0a	0.0a	1.2a	1.2a	0.4a	0.0a	0.0a	4.2a

*Explanations, see Table 1

The beneficial role of FeEDDHA

Chelate FeEDDHA, when added to the regeneration medium, significantly increased the regeneration of all the investigated raspberry cultivars (Tab. 6 and 7). Medium acidity adjustment from pH 4.6 to 5.6 pH did not influence regeneration, with a minor exception for 'Canby', where the lowest pH value increased regeneration, more so in interaction with FeEDDHA. In 'Malling Seedling', an increase of regeneration efficiency was observed using the combinations of FeEDTA + FeEDDHA, at pH 4.6 (Tab. 7).

DISCUSSION

The efficiency of adventitious regeneration is closely related to the capability of selecting valuable genetic variants that are interesting for breeders. The probability to obtain desirable genotypes grows with the number of independently regenerated shoots. It is not clear which stimuli allow some cells to change their developmental destination. Instead of differentiating, they may undergo divisions, leading to the development of a meristematic structure. Subjecting explants to different chemical and physical stimuli, we could not be

Table 6. The influence of Fe source and medium acidity of the regeneration medium on regeneration coefficient of red raspberry (number of responding leaves x number of adventitious shoots per responding leaf); n = 6

Treatment	Beskid	Canby	Malling Seedling	Norna	Veten
FeEDTA	20.4a*	5.7a	2.3a	7.6a	8.0a
FeEDTA + FeEDDHA	27.0a	12.9b	7.0b	11.1a	20.1b
FeEDDHA	35.6b	20.6c	3.3a	16.7b	17.7b
pH 4.6	31.8a	14.3a	5.4a	12.8a	14.4a
pH 5.0	27.7a	14.9a	3.8a	11.3a	16.9a
pH 5.6	23.6a	10.0a	3.4a	11.2a	14.4a

*Explanations, see Table 1

Table 7. Analysis of variance for the regeneration coefficient

Source of variation	Beskid	Canby	Malling Seedling	Norna	Veten
Fe source	**	**	**	*	**
pH	ns	o	ns	ns	ns
Fe source x pH	ns	**	*	ns	ns

sure of how they evoke the sequence of gene expression/suppression, which is necessary to obtain adventitious shoots.

Red raspberry is harder to regenerate than black raspberry and blackberry (McNicol and Graham, 1990; Graham et al., 1997; Mezzetti et al., 1997). The results of our experiments confirmed the large differences in the regenerative ability between cultivars, as earlier indicated by McNicol and Graham (1990), Graham et al. (1997) and Turk et al. (1994).

In this paper, we present results from experiments on some factors, applied to optimize the regeneration of red raspberry. These factors had been earlier recommended in other publications, as increasing the competence for regeneration and/or efficiency of regeneration. It appears that the type and concentration of cytokinin in the regeneration medium, plays a major role. In our experiments, the most effective cytokinin was thidiazuron, at a concentration of 0.1 mg l^{-1}. At higher TDZ concentrations, the number of regenerated shoots did not increase, although excessive hyperhydricity and dying was observed. TDZ was recommended for the regeneration of raspberries using a range of concentrations (in mg l^{-1}), 0.1-1.0 (Mathews et al., 1995); 0.2 (Turk et al., 1994); 1.0-2.0 (Cousineau and Donnelly, 1991; Graham et al., 1997); and 1.1 (Fiola et al., 1990). Other results showed that media containing BAP stimulated the

adventitious regeneration of raspberry (McNicol and Graham, 1990; Hoepfner et al., 1996; Mezzetti et al., 1997). However, the concentration of auxin had a somewhat lesser impact. In our experiments, IBA at 0.1 mg l^{-1}, was shown to give the best results, although for 'Veten', 0.05 mg l^{-1} produced a better result. In comparison to that on media with IBA and IAA, regeneration with NAA (as advised by Graham et al., 1997) was significantly worse. Auxin IBA was applied most often for the regeneration of raspberry, with only Fiola et al. (1990) having omitted auxin from the medium during their research.

MS mineral salts were used most often for the regeneration of raspberry, but Turk et al. (1994) ascertained the usefulness of the N_6 medium. In our experiments, the worst results were obtained with B5 and the best on MS, although 'Beskid', 'Canby' and 'Veten' yielded a similar effect on MS and N_6, and the mineral composition of the regeneration medium had no influence on the regeneration of 'Malling Seedling'.

Results presented here confirmed our earlier finding (Zawadzka and Orlikowska, 2006) that FeEDDHA added to the regeneration medium as the iron source, is especially effective in adventitious regeneration of red raspberry cultivars.

We can say, with a high probability, that the quality of donor cultures is very important for adventitious regeneration. The competence for regeneration had been improved through modification of the type and/or concentration of cytokinin (Welander, 1985) and mineral salts (Welander, 1985; Sobczykiewicz, 1992). Results obtained by Swartz et al. (1990) for raspberry, Tsao and Reed (2002) for blackberry and Kucharska and Orlikowska (2005) for rose, indicate the incubation of donor shoots on a medium with TDZ as being beneficial for regeneration; however, this was generally not confirmed in our experiments. A lack of cytokinin in the donor multiplication medium had the worst effect in our experiment, although McNicol and Graham (1990), Graham et al. (1997) and Millan-Mendoza (1998) advised it for the regeneration of raspberry.

The genetic differentiation of red raspberry cultivars also has its reflection in *in vitro* cultures in the process of adventitious regeneration from leaves. Independently of the employed conditions, 'Beskid' had the highest regeneration potential and 'Malling Seedling' the lowest. The cultivars investigated here reacted differently to experimental conditions. Only the presence of iron in the form of chelate FeEDDHA significantly stimulated the regeneration of all the cultivars, although the level of improvement was also genotype dependent (Zawadzka and Orlikowska, 2006).

CONCLUSIONS

Based on the results of experiments on the regeneration of adventitious shoots from the leaves of five red raspberry cultivars, we recommend growing donor shoots of 'Beskid', 'Norna' and 'Veten' on MS

medium, and of 'Canby' and 'Malling Seedling' on N_6 medium, both containing BAP in the concentration 0.6 mg l^{-1} and 0.1 mg l^{-1} IBA. For regeneration of all cultivars investigated, we recom-mend MS medium, modified by the replacing FeEDTA with the equvalent amount of FeEDDHA (118.2 mg l^{-1}) (Zawadzka and Orlikowska, 2006), and containing 0.1 mg l^{-1} TDZ and 0.1 mg l^{-1} IBA. The exception to this is 'Canby', for which the auxin IAA, at concentration 0.2 mgl^{-1}, is recom-mended. Acidity of regeneration medium for 'Canby' and 'Malling Seedling' should be lowered to 4.6.

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CZYNNIKI MODYFIKUJĄCE REGENERACJĘ *IN VITRO* PĘDÓW PRZYBYSZOWYCH PIĘCIU ODMIAN MALINY CZERWONEJ

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S T R E S Z C Z E N I E

Celem doświadczeń było określenie czynników poprawiających regenerację przybyszową *in vitro* z liści pięciu odmian maliny – 'Beskid', 'Canby', 'Malling Seedling', 'Norna' i 'Veten'. Chelat żelaza – FeEDDHA był czynnikiem wpływającym na zwiększenie zdolności regeneracyjnych wszystkich odmian. Wpływ innych badanych czynników był ograniczony do poszczególnych odmian. Zgodnie z wynikami doświadczeń, pożywka regeneracyjna powinna zawierać sole MS (Murashige i Skoog, 1962), z chelatem FeEDDHA zamiast FeEDTA oraz 0,1 mgl^{-1} tidiazuronu (TDZ) i 0,1 mgl^{-1} kwasu indolilo-3-masłowego (IBA). Dla odmiany 'Canby' lepsze wyniki otrzymano przy zastosowaniu 0,2 mgl^{-1} kwasu indolilo-3-octowego (IAA). Odmiany 'Canby' i 'Malling Seedling' regenerowały lepiej na pożywce o pH obniżonym do 4,6. Rekomendowana pożywka donorowa, z której pobierano liście do regeneracji, powinna być oparta na formule MS dla odmian 'Beskid', 'Norna' i 'Veten', a dla odmian 'Canby' i 'Malling Seedling' korzystniejsza była pożywka N_6 (Chu i wsp., 1975), z benzyloaminopuryną (BAP) jako cytokininą. Niezależnie od zastosowanych czynników, najwydajniejszą regenerację zanotowano dla odmiany 'Beskid', a najniższą dla odmiany 'Malling Seedling'.

Słowa kluczowe: auksyna IBA, BAP, chelat FeEDDHA, sole mineralne, tidiazuron