J. Plant Biotechnology (2005) Vol. 7 (4). pp. 241~246

Journal of Plant Biotechnology

Efficient and Reliable *in vitro* Regeneration System for *Rubus* Species as the Basis of Genetic Engineering

Katalin Kálai[1], Annamária Mészáros[2], Ferenc Dénes[3], József Zatykó[3], Ervin Balázs[1,2*]

[1]Agricultural Biotechnology Center, Szent-Györgyi A. u. 4., H-2100 Gödöllö, Hungary; [2]Agricultural Research Institute of the Hungarian Academy of Sciences, Brunszvik u.2., H-2464, Martonvásár, Hungary; [3]Fertőd Research Institute For Fruit Growing, Kossuth u. 57., H-9435 Sarród, Hungary

Abstract

Factors affecting regeneration of different *Rubus* varieties (blackberry, raspberry and their hybrid) were examined and a reliable regeneration system was established. Media for stock plant maintenance were tested; different explants and media were investigated to find the best circumstances for the regeneration. The effect of the commonly used antibiotics was studied to determine the most suitable one for selection of the transformants. We found that both MS and LS media supplemented by 20 gL^{-1} sucrose are suitable for the stock plant maintenance. The optimal hormone content for the stock plants is 0.125 mgL^{-1} 6-benzylaminopurine (BAP) with 0.01 mgL^{-1} indole-3- butyric acid (IBA). The highest regeneration rate was observed on medium containing MS salts with B5 vitamins complemented with glucose, sucrose, maltose, 10 gL^{-1} each, supplemented with benzylaminopurine riboside (BAR) (2 mgL^{-1}) and indole-3-acetic acid (IAA) (0.1 mgL^{-1}). The regenerated shoots appeared directly from the cut edges, without callus phase. Hygromycin and geneticin proved to be good selection agents for the *Rubus* explants, but due to their severe effect on the tissues we propose to use marker-free constructions for the transformation.

Key words: Tissue culture, *Rubus* species, antibiotics, regeneration conditions

* Corresponding author, E-mail: balazs@abc.hu
Received Feb. 24, 2004; Accepted Nov. 11, 2005

Introduction

Introducing exogenous DNA into crop plants using genetic engineering techniques offers new possibilities to change the genetic make-up of the plant. This method leads for the desired characteristics a lot more quickly than the conventional breeding. The most frequently and successfully used genetic engineering system for the dicotyledonous species is the *Agrobacterium tumefaciens* mediated transformation. There are prerequisites for the efficient plant transformation, such as techniques for regeneration of whole plants from somatic tissues, a suitable gene-vector system and a selection system for identifying the transformants. In this study we focused on achieving a reliable regeneration method in which the whole plant can be regenerated from cells capable to receive the foreign DNA.

The temperate climate is suitable for growing several fruit crops. Like other fruits the production of berries has long history in the Carpathian basin. Due to the historical and economical significance *Rubus* species were chosen as the subject of our work.

Scientific improvements have been involved in the production of berries. Micropropagation has an important role in the production of nursery plants. Virus free stocks are also maintained by sterile cultures. *Rubus* species (raspberry, blackberry and their hybrids) were known to be recalcitrant in the regeneration studies, but in the early nineties several successful trials were published. Since the leaves and the petioles are known to be the most suitable explants for the *Agrobacterium* mediated transformation, we concentrated on these studies (Fiola et al. 1990; Swartz et al. 1990; Owens y de Novoa (Conner 1992;

Cousineau (Donnelly 1991; McNicol (Graham 1990; Turk et al. 1994).

The first aim of our work was to examine the factors are necessary to develop protocols with high regeneration frequencies from leaf and petiole explants of different *Rubus* genotypes. It is a well known fact that great differences can be found among the genotypes, and it is almost impossible to develop a repeatable protocol suitable for all varieties.

For the early selection of the transformants different marker genes are used. The most widespread ones are the herbicide and the antibiotic-resistance markers. These genes are built into the vector construction. The most commonly used antibiotics (kanamycin, geneticin, hygromycin) were examined to determine the tolerance of plant tissues against the selection agents.

Materials and methods

Plant material

One variety of blackberry 'Hull Thornless' (Figure 1A), one variety of hybridberry 'Fertődi Bőtermő' (blackberry x raspberry) (Figure 1B) and two varieties of raspberry 'Malling Exploit' and 'Fertődi 6585/69' (Figure 1C) were involved in our experiment. The basic, virus free shoot culture materials were maintained in the gene bank of the Fertőd Research Institute For Fruit Growing, Sarród.

Micropropagation

Stock plants were selected from a virus free plantation and the sterile cultures were initiated from meristems. Shoot cultures were maintained on medium with MS salts and vitamins (Murashige & Skoog, 1962), or MS salts and B5 vitamins (Gamborg et al. 1968) or on modified LS (James et al. 1980) media. The LS medium was modified in ferric content, since in our experiments 50 mgL^{-1} FeNaEDTA was used. All media were supplemented with 20 gL^{-1} sucrose and 0.01 mgL^{-1} IBA. To obtain the best rate of proliferation and the best quality plants different concentrations of BAP were tested. BAP was chosen because most of the papers suggest this cytokinin for maintaining and proliferation. The stock plants were maintained in different containers: plastic boxes or plastic round vessels with breathing strip, designed for tissue cultures (products of Duchefa) and in 500 mL or 100 mL glass flasks in order to find the most appropriate vessel. The growing conditions as follows: 25℃ light (16h) /20℃ dark (8h) regime, with 30 $\mu mol\ m^{-2}\ sec^{-1}$ light intensity.

Figure 1. Varieties used in the experiments. A: blackberry 'Hull Thornless'; B. hybridberry 'Fertődi Bőtermő', C: raspberry 'Malling Exploit'

Regeneration experiments

The apical three leaves were excised from the 4 week old shoots grown on proliferation media. In some experiments the leaves with a small petiole segment were detached and two or three transversal cuts were made across the leaf midrib before the leaves were placed adaxial side down on the medium. In other cases the leaves were cut into two parts: one third of the leaf with the petiole segment and the apical two-third of the leaves. Here the cut edges were attached to the surface of medium. In one experiment, discs were excised from the leaves with a sterile cork borer and they were placed adaxial side down on the media. The petiole explants also were tested: 0.5-1 cm long petiole segments were placed on the surface of the media.

To obtain the optimal growth conditions different media were tested. The media in all experiments contained MS salts, B5 vitamins, they were solidified with 0.65% Oxoid agar, and their pH was adjusted to 5.6 with KOH before autoclaving. They varied in the concentrations and combinations of carbohydrates (glucose, sucrose, maltose), auxins and cytokinins (See Table 1). The cultures were maintained under 25℃ light (16h) / 20℃ dark (8) regime.

Regeneration efficiency in the tables refers the number of the regenerating explants correlated to the number of explants in the trial. Usually 80 explants/ variety/ hormone combination were used in three repetitions. Only the rooted shoots were counted. From one explant several shoots are obtained, but this effect was not considered in the statistics.

The effect of the different antibiotics

In the first experiment we investigated the effect of the kanamycin on shoot growth. The shoot tips were placed on the proliferation media containing 0, 20, 40, 50, 60 mgL^{-1} kanamycin. The antibiotic was filter sterilised and added to the media cooled below 55℃.

In a separate experiment leaves with transversal cuts across their midrib were placed on the medium D with

Table 1. The types and concentrations of growth regulators used. The media contains MS salts, B5 vitamins and glucose, sucrose, maltose, 10gL^{-1} each, unless otherwise stated (a: thidiazuron, b: 2-isopentenyl-adenine)

Sign	TDZ [a] (mgL^{-1})	BAP (mgL^{-1})	BAR (mgL^{-1})	2ip [b] (mgL^{-1})	IAA (mgL^{-1})	IBA (mgL^{-1})
A	1	1	1		0.1	
B	1	1		1	0.1	
C			2		0.1	
D		2				0.1
E	2.2					

different concentrations of kanamycin (0, 10, 20, 40, 60, 100, 150 mgL^{-1}), hygromycin (0, 10, 15, 20, 40 mgL^{-1}) and geneticin (0, 5, 10, 20, 30, 40 mgL^{-1}).

Results and Discussion

The main goal of this study was to develop an effective regeneration system, which can be the basic of the *Agrobacterium* mediated transformation. Good quality stock plants are needed for it. We observed that *Rubus* plants need different media for maintenance. During the culture periods, several media were examined and seemed promising, but as time went none of them was satisfactory. The solution for this problem was the changing of the media. By the alternation of MS and modified LS media good quality stock plants can be maintained. We found that considerable amount of micropropagated shoots in the best quality can be gained and maintained on the described media supplemented with 0.125 mgL^{-1} BAP. The higher cytokinin concentrations yielded better shoot multiplication rate, but the leaves of the new shoots were too small for the further use. This very low cytokinin rate converges to Graham et al. (1997) results, in which no growth regulators were used for the proliferation. Although Mathews (1995) maintained the shoot cultures on media containing 1 mgL^{-1} BAP we found it too much. Hoepfner (1989) found no significant difference for the multiplication rate between 0.5 and 2 mgL^{-1} BAP. In order to maintain the juvenile stage, shoots should be transferred to fresh medium in every 4 weeks.

Stock plants need big airspace vessels. In small vessels the leaves of the plants became soft, which is not suitable for *Agrobacterium* mediated transformation. For this reason 500 mL flask and round vessels from Duchefa was used in our experiments.

During the preliminary regeneration trials the best results were obtained with Fertődi Bőtermő, so this variety was used for the optimisation experiments. In these trials, medium D was used. First the explant-types were tested. The results (Table 2) show that the whole leaves with transversal cuts on the midrib and the upper part of the halved leaves proved to be the best explants. Regeneration was not observed on the petiole segments, although Mezzetti et al. (1997) found the petiole segments suitable for the regeneration. Usually the whole leaves were used in the previously published experiments (Fiola et al. 1990; Turk et al 1994; Mezzetti et al. 1997; Swartz et al. 1990). The leaf-disc method reported by McNicol and Graham (1989) was also tested and resulted several regenerants.

Table 2. The effect of the explant type on the regeneration of Fertődi Bőtermő

Explant-types	Regeneration efficiency (%)
Whole leaf with transversal cut	41
Halved leaf, apical part	26
Halved leaf, shoulder part	10
Leaf-disc	17
Petiole segment	0

Table 3. The effect of the sugar in the media on the regeneration of Fertődi Bőtermő

Type of sugars in the medium	Regeneration efficiency (%)
Sucrose (20gL^{-1})	12.5
Glucose (20gL^{-1})	18
"mix"	25

In order to optimise the sugar content for the regeneration we compared media containing glucose (20 gL^{-1}), sucrose (20 gL^{-1}), or the mixture ("mix") of glucose, sucrose and maltose (10 gL^{-1} each) (Csányi et al. 1999). The effect of the different carbohydrates on the regeneration of *Rubus* has not been studied previously. The results of the experiment (Table 3) indicate that the best composition for the regeneration contains the "mixed" sugars, although the application of glucose proved to be satisfying too.

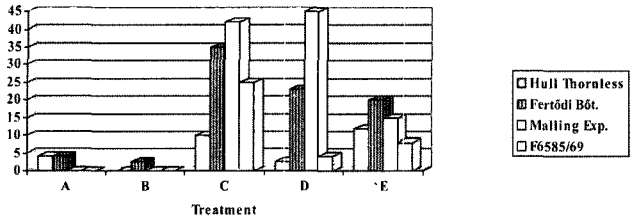

Figure 2. The effect of the growth regulators on the regeneration. Treatments: A: 1 mgL^{-1} BAP+ 1 mgL^{-1} BAR+ 1 mgL^{-1} TDZ+ 0.1 mgL^{-1} IAA ; B: 1 mgL^{-1} BAP+ 1 mgL^{-1} 2ip+ 1 mgL^{-1} TDZ+ 0.1 mgL^{-1} IAA ; C: 2 mgL^{-1} BAR+ 0.1 mgL^{-1} IAA ; D: 2 mgL^{-1} BAP+ 0.1 mgL^{-1} IBA ; E: 2.2 mgL^{-1} TDZ

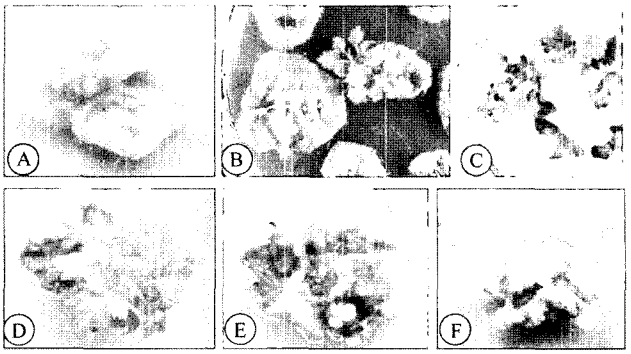

Figure 3. Regenerating shoots: A-B: Direct organogenesis on Fertődi Bőterő leaves; C, F: New shoots on Fertődi 6585/69 leaves; D: Regenerants on Hull Thornless explants; E: Callus induction on Hull Thornless explants, from which shoots cannot be regenerated

In the growth regulator experiment we used all cultivars, because the different genotypes usually have different requirements. The results are shown in Figure 2. It can be stated that combining the different cytokinins does not have positive effects on the regeneration. On the other hand combining one kind of cytokinin with an auxin resulted regeneration in all cultivars. This observation corresponds with the results of Popescu and Isac (2000). BAR with IAA and BAP with IBA proved to be the best combinations in our experiments. The application of BAR for regenerating *Rubus* has not been mentioned in the literature previously. The BAP-IBA combination was advocated by other authors (Mezzetti et al. 1997; Graham and McNicol 1989).

Some differences were obtained between the genotypes. Hybridberry 'Fertődi Bőtermő" regenerated on each media, although the efficacy was very low for medium A and B. Media C was better, than D or E for this cultivar (Figure 3a-b). The regeneration rate of Malling Exploit seemed outstanding on medium containing BAR+IAA; but media D also was suitable for this variety. For Fertődi 6585/69 media D contains the best hormone-content (Figure 3c, f). The weakest response for the treatments was observed in the case of blackberry 'Hull Thornless', but the thidiazuron (TDZ) seemed to have the best effect on this cultivar (Figure 3d). Hull Thornless explants are susceptible to strong callus formation, but from these calli shoots cannot be regenerated (Figure 3b).

In our experiments the effect of TDZ was different than in the literature. According to several authors (Graham et al. 1997; Hassan et al. 1993; Cousineau (Donnelly 1991; Swartz et al. 1990; Turk et al, 1994) TDZ - alone or combined with auxines - results higher regeneration rates than any other cytokinin. In our experiments good results obtained by using TDZ, but the shoots were usually bushy and small, rooting them was difficult. The rooting difficulties resulted the lower regeneration efficiency shown in Figure 2, because in the statistics only the rooted plantlets were considered. Other drawback of TDZ is that the large number of shoots occurred from one cell, which is disadvantageous during the transformation. Regenerants from different cells increase the prospect of occurring transgenic plantlets.

Testing the tolerance of plant tissues against the commonly used antibiotics was one of the aims of our work. Since our experiments are to substantiate an *Agrobacterium* mediated transformation system, our purpose was to estimate the effect of the most commonly used antibiotics on the plant tissues. We wanted to ascertain which antibiotic is the most suitable for the selection. Previously only data concerning the kanamycin tolerance were reported by Fiola et al. (1990). They found that 50 mgL^{-1} kanamycin completely inhibited the shoot organogenesis. The effect of kanamycin on shoots was tested in our laboratory too. It was shown that the effect was not the same on the different varieties. Generally it can be stated that kanamycin caused whitening first on the younger leaves, along the veins. In higher concentration the whole shoot tip became white (See Figure 4). The highest tolerance against kanamycin was observed on hybridberry plants. While on the other three cultivars serious damage can be seen already at 40 mgL^{-1} concentration, the hybridberry 'Fertődi Bőtermő' is able to survive the 50 mgL^{-1} kanamycin.

In a separate experiment the effect of three antibiotics; kanamycin, hygromycin and geneticin were tested. They were added to the media in different concentrations. Results are shown on figure-series 4. It could be observed that hygromycin has the most serious effect on each cultivar. It is extremely toxic for the raspberry and blackberry leaves at the concentration of 20 mgL^{-1} (Figure 5a). The hybridberry leaves suffers of yellowing only at this concentration, but they are harmed seriously at 60 mgL^{-1} (photo not shown). Geneticin also damages the leaves at low concentration. A medium containing 20 mgL^{-1} of geneticin causes lesions on

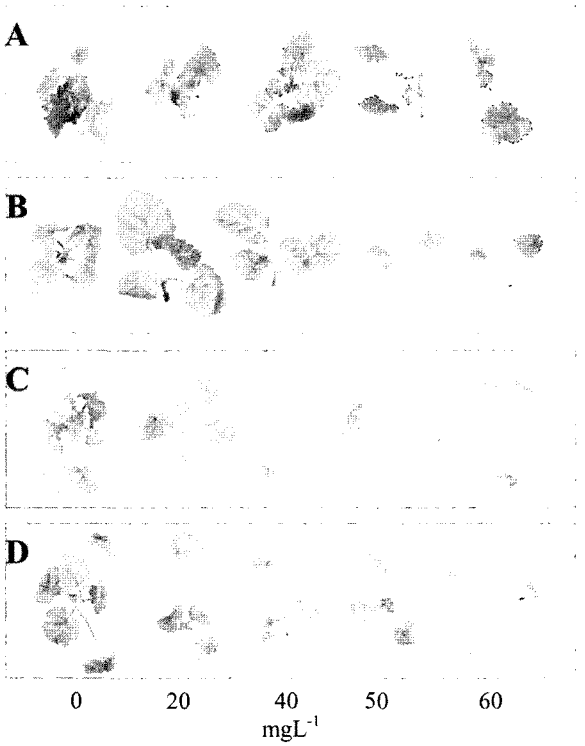

Figure 4. The effect of kanamycin on *Rubus* shoots. A: Hull Thornless; B: Fertődi Bőtermő; C: Malling Exploit; D: Fertődi 6585/69

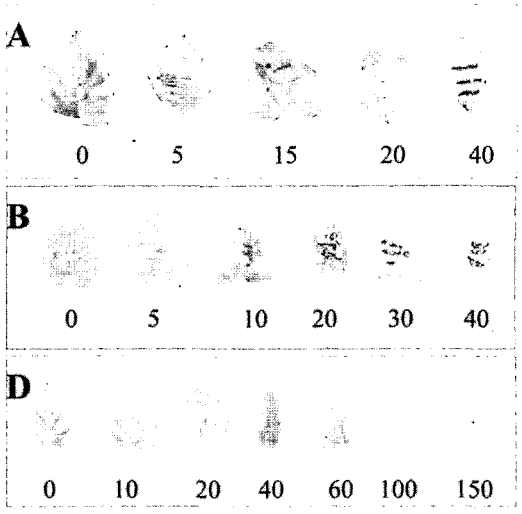

Figure 5. The effect of the different antibiotics on *Rubus* leaves. The numbers show the concentration of the different antibiotics in mgL^{-1}. A: Hygromycin on Hull Thornless leaves. B: Geneticin on Malling Exploit leaves. C: Kanamycin on Fertődi Bőtermő leaves.

the leaves around the wounds. Kanamycin is not as harsh; at lower concentration it only induces yellowing. Lesions are occurred at about 60 mgL^{-1} concentration.

Summarising our results it can be asserted that a direct organogenesis method was established. The medium suitable for each examined cultivar contains MS salts, B5 vitamins, glucose, sucrose, maltose, 10gL^{-1} each, 2mgL^{-1} BAR and 0.1 mgL^{-1} IAA. The best explants are the whole leaves with cuts on the midrib or the halved leaves. This method works for different species and could be a basis of an *Agrobacterium tumefaciens* mediated transformation. We also found that hygromycin and geneticin are good selection agents for the *Rubus* species even in low concentration. Kanamycin can be suitable for the selection, but in a higher concentration, which otherwise can set back the regeneration. Since all of them have very strong effect on the explants, we suggest using the marker-free transformation constructions.

One comment should be added. Different genotypes have different requirements. When establishing a regeneration system this has to be considered. Our experiments show the different aspects of regeneration and give ideas how to deal with the recalcitrant *Rubus* varieties and which ways are worth following to reach the success.

Acknowledgements

This work was supported by T 032402 OTKA grant and OTKA Science Schools (TS 44778). Special thanks are due to Márta Csányi for the perfect and conscientious assistance.

References

Cousineau JC, Donnelly DJ (1991) Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell Tiss Org Cult 38: 11-17

Csányi M, Wittner A, Nagy Á, Balla I, Vértesy J, Palkovics L, Balázs E (1999) Tissue culture of stone fruit plants basis for their genetic engineering. J Plant Biotech 1: 91-95

Fiola JA, Hassan MA, Swartz HJ, Bors RH, McNicol R (1990) Effect of thidiazuron, light fluence and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tiss Org Cult 20: 223-228

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension culture of soybean root cells. Exp Cell Res 50:151-158

Graham J, McNicol RJ (1989) Planlet regeneration and genetic transformation of soft fruit species. Acta Hort 280: 517-522

Graham J, Iasi L, Millam S (1997) Genotype-specific regeneration from a number of *Rubus* cultivars. Plant Cell Tiss Org Cult 48: 167-173

Hassan MA, Swartz HJ, Inamine G, Mullineaux P (1993) *Agrobacterium tumefaciens*-mediated transformation of several *Rubus* genotypes and recovery of transformed plants.

Plant Cell Tiss Org Cult 33: 9-17

Hoepfner A. (1989) *In vitro* propagation of red raspberry. Acta Hort 262: 285-288

James DJ, Knight VH, Thurbon IJ (1980) Micropropagation of red raspberry and the influence of phloroglucinol. Scientia Hort 12: 313-319

Mathews H, Wagoner W, Cohen C, Kellogg J, Bestwick R (1995) Efficient genetic transformation of red raspberry *Rubus idaeus*. Plant Cell Rep 14:471-476

Mezzetti B, Savini G, Carnevali F, Mott D (1997) Plant genotype and growth regulators interaction affecting *in vitro* morphogenesis of blackberry and raspberry. Biol Plant 1: 139-150

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 437-497

Owens y de Novoa C, Conner AJ (1992) Comparison of *in vitro* shoot regeneration protocols from *Rubus* leaf explants. N Z J Crop Hort Sci 20: 471- 476

Popescu AN, Isac V (2000) High frequency shoot regeneration from leaf-derived callus in raspberry (*Rubus idaeus* L.) Acta Hort 538: 667-672

Swartz HJ, Bors R, Mohamed F, Naess SK (1990) The effect of *in vitro* pretreatments on subsequent shoot organogenesis from excised *Rubus* and *Malus* leaves. Plant Cell Tiss Org Cult 21: 179-194

Turk BA, Swartz HJ, Zimmerman RH (1994) Adventitious shoot regeneration from *in vitro*-cultured leaves of *Rubus* genotypes. Plant Cell Tiss Org Cult 381: 11-17