



IMPROVEMENT OF MEDIUM FOR *MISCANTHUS* × *GIGANTEUS* CALLUS INDUCTION AND PLANT REGENERATION

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Miscanthus × *giganteus* tissue cultures brown quickly and strongly, probably as an effect of oxidation of phenolics produced by plant tissue. The aim of the work was to improve the medium composition for callus induction and plant regeneration of *M. giganteus*, with two experiments. The first one was aimed at developing a protocol to inhibit biosynthesis and oxidation of phenolics. Callus was induced from immature inflorescences on basal MS medium with 6.5 mg · dm⁻³ 2,4-D, 0.25 mg · dm⁻³ BAP, 500 mg · dm⁻³ casein hydrolysate and 30 g · dm⁻³ sucrose (control medium), and this medium supplemented with one of the following: 200 mg · dm⁻³ chitosan, 65 g · dm⁻³ banana pulp (BP), 100 mg · dm⁻³ cysteine, or 30 g · dm⁻³ honey instead of sucrose. Plant regeneration used basal MS medium supplemented with 30 g · dm⁻³ sucrose and 0.2 mg · dm⁻³ BAP or 0.05 mg · dm⁻³ KIN. The second experiment was to verify whether BP and honey increase callus production and plant regeneration. For callus induction the explants were put on the control medium and medium supplemented with one of these: 65 g · dm⁻³ BP, 30 g · dm⁻³ honey instead of sucrose, or 65 g · dm⁻³ BP + 30 g · dm⁻³ honey instead of sucrose. The regeneration medium was basal MS medium supplemented with 0.05 mg · dm⁻³ KIN and 30 g · dm⁻³ sucrose or 30 g · dm⁻³ honey. Tissue browning was independent of medium content, but there was less browning on medium supplemented with honey. In the first experiment, regenerated plants were obtained only on basal MS medium with 30 g · dm⁻³ sucrose and 0.05 mg · dm⁻³ KIN. In the second experiment, MS medium containing BP and honey instead of sucrose was the best medium for callus induction, and regeneration from these calli was highest on basal MS with KIN and honey.

Key words: banana pulp, chitosan, cysteine, embryo-like structures, honey, *Miscanthus*, phenolic oxidation, regenerants, tissue browning.

INTRODUCTION

Miscanthus × *giganteus* is a sterile triploid (hybrid of diploid *Miscanthus sinensis* and tetraploid *M. sacchariflorus*) which naturally reproduces vegetatively from rootstocks. This grass is sometimes used as an ornamental plant in gardens, and has attracted the interest of producers because it could be used as a biofuel or a cellulose source. *Miscanthus* is a C₄ plant, characterized by high CO₂ absorption, high yield potential and low water consumption. It can be cultivated on poor soils (Lewandowski, 2006). To use *Miscanthus* plants as an energy source requires about 10,000 seedlings per hectare. *Miscanthus* × *giganteus* is a thermophilous plant; its reproduction proceeds efficiently at temperatures above 20°C (Naidu and Long, 2004; Lewandowski, 2006). These thermal condi-

tions significantly increase production costs. By in vitro technique it is possible to obtain many regenerants from a small culture area. Tissue cultures are usually characterized by high efficiency of regeneration, with multiple plantlets produced from one callus. Micropropagation is now commonly applied in agriculture and horticulture. Using this technique about 800 million plants are produced per year worldwide, roughly 90% of them ornamentals (Debergh, 1994).

Callus induction from immature inflorescences of *Miscanthus* involves the serious problem of very intensive phenolic compound production by explants (Płażek et al., 2007). The products of phenolic oxidation are toxic to callus tissue and reduce

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Abbreviations: AOA – α-aminooxyacetic acid; BP – banana pulp; BAP – 6-benzylaminopurine; ELS – embryo-like structures; MS – basal medium of Murashige and Skoog (1962); PAL – phenylalanine ammonia-lyase; PPF – photosynthetic photon flux density; PVP – polyvinylpyrrolidone.

its growth. They cause tissue browning 2–3 days after each passage of explants. Supplementation of callus induction medium with α -aminooxyacetic acid (AOA), an inhibitor of phenylalanine ammonia-lyase (PAL), the key enzyme of the phenylpropanoid pathway, and with polyvinylpyrrolidone (PVP) did not decrease phenolic production and secretion to the medium (Lewandowski and Kahnt, 1993; Płażek et al., 2007).

The problem of callus induction and tissue browning in *Miscanthus* culture is hardly documented in the literature. Lewandowski and Kahnt (1993) showed that adding compounds such as ascorbate or charcoal to the medium did not limit accumulation of phenolics. Holme et al. (1997) reported that proline added to the medium increased the efficiency of *Miscanthus* embryogenic callus induction from immature inflorescences, but did not mention tissue browning.

Tissue browning caused by phenolic oxidation is very often observed in cultures of many plant species. To reduce it, various compounds are usually added to media for callus induction or plant regeneration. The compounds most often used include chitosan, cysteine, phenylpropanoid pathway inhibitors, chelators that inactivate metal ions needed for enzyme-catalysed oxidation of phenols, antioxidants such as ascorbate, or absorbents such as charcoal. Chitosan has antimicrobial properties, is non-toxic and has been shown to inhibit enzymatic browning in food (Sapers, 1992). It shows potential inhibitory activity against polyphenol oxidase and peroxidase activity in fruit (Zhang and Quantick, 1997). Cysteine is an effective inhibitor of enzymatic browning and has also been shown to reduce *o*-quinones to their phenol precursors (Walker, 1977; Cilliers and Singleton, 1990). Honey and some fruits (coconut, banana) are natural sources of various hormones and compounds such as vitamins, mineral salts, and many phenolic compounds with various properties. These compounds are used as supplements for callus induction or organ differentiation media. Honey contains antioxidative phenolics (flavonoids, phenolic acids) and ascorbate which prevents tissue browning (Kahn, 1985; Hołderna-Kędzia and Kędzia, 2006). Honey peptide demonstrates an inhibitory effect on polyphenol oxidase activity by chelating essential copper at the active site of polyphenol oxidase (Kahn, 1985). Banana pulp is a rich source of natural cytokinins which inhibit culture initiation and promote differentiation and growth of shoots at later stages (Seeni and Latha, 2000). Other hormones such as auxins and gibberellins have also been found in banana fruit (Chugh et al., 2009).

In the present work we sought to optimize the composition of medium for callus induction and plant regeneration of *Miscanthus* \times *giganteus*, in

two experiments. In the first one the callus-inducing medium was supplemented with compounds that inhibit phenolic oxidation, such as chitosan, cysteine and honey. The second one was done to see whether banana pulp and honey increase callus production and plant regeneration. The results from experiment 1 were the basis for planning experiment 2.

MATERIALS AND METHODS

EXPERIMENT 1

Callus was induced from immature inflorescences collected from donor plants grown in a greenhouse at 24°C in daylight under a 16 h photoperiod with light intensity 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (photosynthetic photon flux density). Inflorescences 10–20 cm long were cut at flag leaf stage, then sterilized with 70% ethanol for 1 min and with sodium hypochlorite (Domestos) diluted in distilled water (1:10 v:v) for 10 min. Then they were washed three times in sterile water, cut into 0.5 cm pieces and put on basal MS (Murashige and Skoog, 1962) medium (SIGMA) containing 6.5 $\text{mg} \cdot \text{dm}^{-3}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 $\text{mg} \cdot \text{dm}^{-3}$ 6-benzylaminopurine (BAP), 500 $\text{mg} \cdot \text{dm}^{-3}$ casein hydrolysate and 30 $\text{g} \cdot \text{dm}^{-3}$ sucrose (this medium served as the control medium), and on this medium supplemented with one of the following: 200 $\text{mg} \cdot \text{dm}^{-3}$ chitosan, 65 $\text{g} \cdot \text{dm}^{-3}$ banana pulp (BP), 100 $\text{mg} \cdot \text{dm}^{-3}$ cysteine, or 30 $\text{g} \cdot \text{dm}^{-3}$ honey (commercial, polyfloral) used instead of sucrose. Banana pulp was obtained from blended commercial banana fruit. The pH of the media was adjusted to 5.6–5.8 before adding 0.8% agar (SIGMA). The media were autoclaved at 121°C at 0.1 MPa for 20 min. Callus was induced at 25°C in the dark. Individual calli grown on rapidly browning explants were passaged twice on fresh media with the same composition.

After 2 months, white calli were passaged to magenta vessels containing regeneration medium, that is, basal MS medium supplemented with 30 $\text{g} \cdot \text{dm}^{-3}$ sucrose and 0.2 $\text{mg} \cdot \text{dm}^{-3}$ BAP or 0.05 $\text{mg} \cdot \text{dm}^{-3}$ kinetin (KIN). Regeneration proceeded at 20°C at 300 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PPFD. The regenerants were transferred first to small pots (7 cm diam.) with sterile perlite for 1 week (the plantlets were covered with glass to maintain high humidity) and grown under the same conditions as during regeneration, and subsequently were repotted to pots (10 cm diam.) with a mixture of soil:peat:sand (2:2:1 v/v/v) at pH 5.8. and grown in the greenhouse under daylight at 20°C.

EXPERIMENT 2

Callus was induced from immature inflorescences as described in experiment 1. Explants were put on

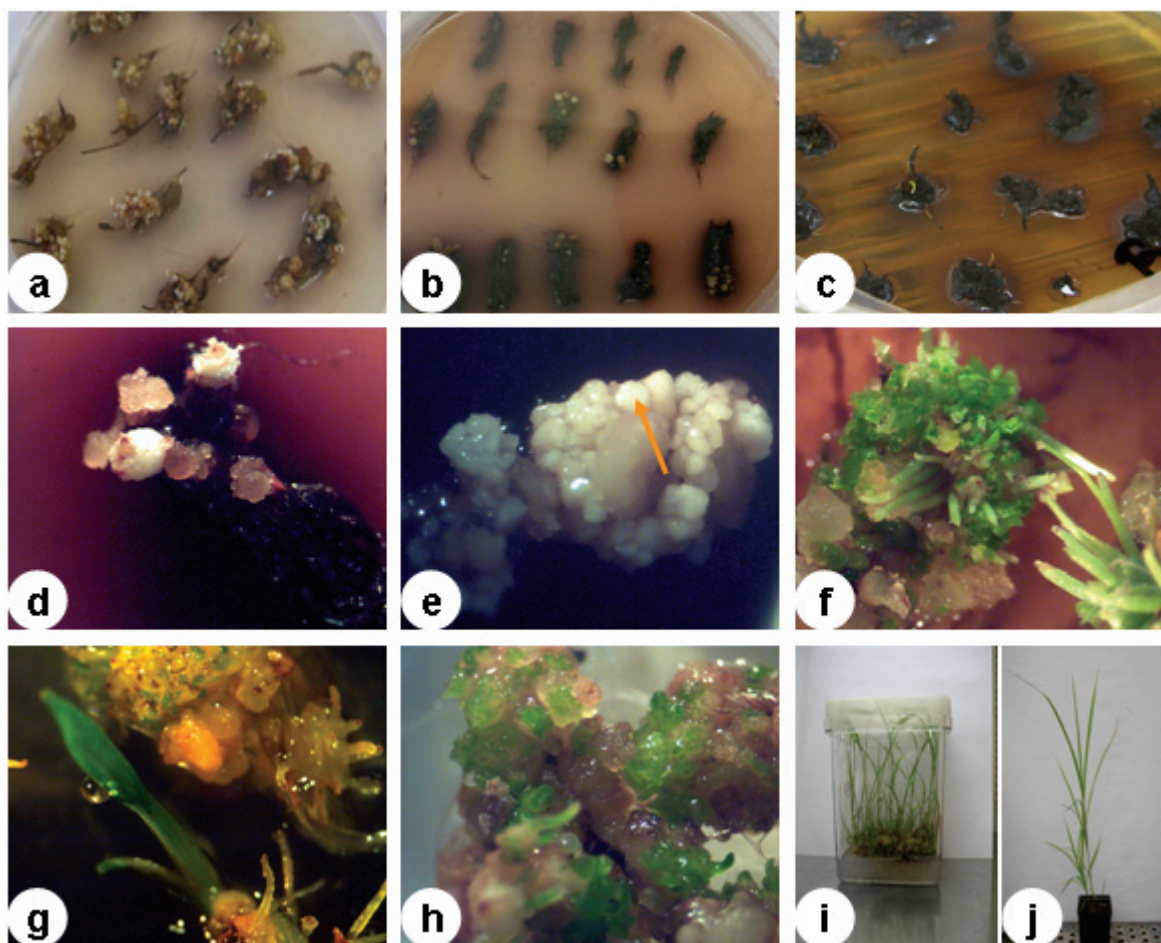


Fig. 1. Callus induction and plant regeneration of *Miscanthus × giganteus*. (a) Callus initiation on MS medium with 30 g dm^{-3} honey instead of sucrose, (b) Severe browning of explants (immature inflorescences cut into 0.5 cm pieces) on MS with $200 \text{ mg} \cdot \text{dm}^{-3}$ chitosan and (c) with $100 \text{ mg} \cdot \text{dm}^{-3}$ cysteine, (d) Callus developing on dark browning explants, (e) Spongy callus with many white embryo-like structures (arrowed), (f) Start of regeneration on MS with $65 \text{ g} \cdot \text{dm}^{-3}$ BP (many roots greening in light), (g) Leaf regeneration on MS with $0.05 \text{ mg} \cdot \text{dm}^{-3}$ KIN, (h) Leaf buds on MS with $0.2 \text{ mg} \cdot \text{dm}^{-3}$ BAP, (i) Regenerated plantlets on MS with $0.05 \text{ mg} \cdot \text{dm}^{-3}$ KIN, (j) Regenerated *Miscanthus* plant in soil.

MS control medium described in experiment 1 and on this medium supplemented with one of the following: $65 \text{ g} \cdot \text{dm}^{-3}$ BP, $30 \text{ g} \cdot \text{dm}^{-3}$ honey instead of sucrose, or $65 \text{ g} \cdot \text{dm}^{-3}$ BP + $30 \text{ g} \cdot \text{dm}^{-3}$ honey instead of sucrose. To check whether honey and BP significantly changed the osmotic potential of the media, it was measured with a Marcel os.3000 osmometer (Merzet, Warsaw, Poland).

After 2 months of culture the induced calli were directly, without intermediate passages, put on regeneration medium, that is, basal MS medium supplemented with $0.05 \text{ mg} \cdot \text{dm}^{-3}$ of KIN and $30 \text{ g} \cdot \text{dm}^{-3}$ sucrose or $30 \text{ g} \cdot \text{dm}^{-3}$ honey. Calli from each dish were transferred on both regenerating media. The plants were regenerated in conditions as described in experiment 1.

DATA ANALYSIS

The effect of medium content on the number of induced calli and their regeneration ability was analyzed using the Pearson χ^2 test at $p < 0.05$.

RESULTS

EXPERIMENT 1

After 2–4 days of culture the explants darkened, probably as an effect of phenolic accumulation and oxidation. The black products were secreted into the media, forming black grease around the explants. Tissue browning was independent of medium content, but browning was less on medium supplemented with

TABLE 1. Efficiency of callus induction and plant regeneration on MS medium supplemented with: (1) 200 mg dm^{-3} chitosan; or (2) 65 $\text{g} \cdot \text{dm}^{-3}$ BP; or (3) 100 $\text{mg} \cdot \text{dm}^{-3}$ cysteine; or (4) 30 $\text{g} \cdot \text{dm}^{-3}$ honey instead of sucrose

Callus induction medium	No. of explants put on medium	% of explants producing callus	% of regenerating calli	No. of regenerants
0. MS – control	310	84.2	53.9	-
1. MS + chitosan	375	81.3	75.6 **	-
2. MS + BP	215	70.7 *	39.2 *	52
3. MS + cysteine	375	70.4 *	79.1 ***	-
4. MS + honey	600	69.7 *	84.0**	-

Significant differences in frequency versus control (χ^2 test) are asterisked: (p<0.05); ** – p<0.01; *** – p<0.001.

TABLE 2. Osmolality [$\text{mOsm} \cdot \text{kg}^{-1}$] of media applied in the study: MS medium containing 6.5 $\text{mg} \cdot \text{dm}^{-3}$ 2,4-D, 0.25 $\text{mg} \cdot \text{dm}^{-3}$ BAP, 500 $\text{mg} \cdot \text{dm}^{-3}$ casein hydrolysate and 30 $\text{g} \cdot \text{dm}^{-3}$ sucrose as control, and the same medium supplemented with: (1) 65 $\text{g} \cdot \text{dm}^{-3}$ BP; or (2) 30 $\text{g} \cdot \text{dm}^{-3}$ honey instead of sucrose; or (3) 65 $\text{g} \cdot \text{dm}^{-3}$ BP and 30 $\text{g} \cdot \text{dm}^{-3}$ honey instead sucrose

Callus induction medium	$\text{mOsm} \cdot \text{kg}^{-1}$
MS – control	0.273
MS + BP	0.371
MS + honey	0.266
MS + BP + honey	0.364

honey (Fig. 1a–c). After another two weeks the first small calli developed on the explants. The calli were covered with many white embryo-like structures (ELS) (Fig. 1d). The white parts of the calli were separated from the dark tissue and passaged onto fresh media, but this manipulation caused further browning of the callus tissue.

After two months the explants on all types of media were covered with callus producing many ELS (Fig. 1e). Callus induction efficiency was highest on the control MS medium (84.2% explants producing callus) and the medium supplemented with chitosan (81.3%) (Tab. 1). Similar numbers of explants produced calli with ELS on MS medium with cysteine (70.4%), BP (70.7%) or honey (69.7%). Callus induction efficiency on these media was significantly lower than on the control medium. Many roots were induced on MS medium with BP (Fig. 1 f). They greened soon after being transferred to light. The number of calli demonstrating initial regeneration was highest on medium with honey (84%) or cysteine (79.1%). This effect was significantly greater than on the control medium (54%). The number of regenerating calli was lowest on medium supplemented with BP (39.2%), but these calli were the only ones that quickly regenerated whole plants (52 plants); that occurred only on medium with 0.05 $\text{mg} \cdot \text{dm}^{-3}$ of KIN (Fig. 1 g), and no plant regen-

eration was observed on medium containing 0.2 $\text{mg} \cdot \text{dm}^{-3}$ of BAP. The process was stopped at the leaf bud stage (Fig. 1 h). Complete regeneration took 9–11 weeks (Fig. 1 i,j).

EXPERIMENT 2

The osmolality values of the media are given in Table 2. The osmolality of the control MS medium containing 30 $\text{g} \cdot \text{dm}^{-3}$ sucrose ($0.273 \text{ mOsm} \cdot \text{kg}^{-1} \cdot ^\circ\text{C}^{-1}$) and the same medium supplemented with 30 $\text{g} \cdot \text{dm}^{-3}$ honey instead of sucrose ($0.266 \text{ mOsm} \cdot \text{kg}^{-1} \cdot ^\circ\text{C}^{-1}$) was very similar. The addition of banana pulp increased it both in the MS medium with sucrose ($0.371 \text{ mOsm} \cdot \text{kg}^{-1} \cdot ^\circ\text{C}^{-1}$) and in the MS medium with honey ($0.364 \text{ mOsm} \cdot \text{kg}^{-1} \cdot ^\circ\text{C}^{-1}$).

Explant browning was observed on all the applied types of media. As in experiment 1, it was less on MS supplemented with honey instead of sucrose. Calli with ELS developed on the explants independently of the medium content. Callus induction effectiveness was higher on MS supplemented with BP + honey than on the control medium, MS with BP, or MS with honey instead of sucrose (Tab. 3). In this experiment the small calli developing on browning explants were not passaged. The first transfer of calli was done 2 months after the start of culture, directly on the regeneration media.

Apart from MS with honey, the type of induction medium influenced neither the regeneration rate nor the number of plants regenerated on MS with KIN and sucrose (Tab. 3). Calli from MS medium with honey regenerated significantly fewer plants than calli initiated on other media (data analyzed versus number of calli put on regeneration medium). On regeneration MS medium with KIN and honey, regeneration effectiveness was highest in calli induced on both BP-supplemented media. Calli induced on medium with honey or with sucrose (control medium) regenerated similar numbers of plants.

Comparison of the osmolality values of the callus induction media indicates that callus initiation

TABLE 3. Efficiency of callus induction and plant regeneration of *Miscanthus × giganteus*. Callus was induced on control medium MS containing $6.5 \text{ mg} \cdot \text{dm}^{-3}$ 2,4-D, $0.25 \text{ mg} \cdot \text{dm}^{-3}$ BAP, $500 \text{ mg} \cdot \text{dm}^{-3}$ casein hydrolysate and $30 \text{ g} \cdot \text{dm}^{-3}$ sucrose, and on this same medium supplemented with: (1) $65 \text{ g} \cdot \text{dm}^{-3}$ BP; or (2) $30 \text{ g} \cdot \text{dm}^{-3}$ honey instead of sucrose; or (3) $65 \text{ g} \cdot \text{dm}^{-3}$ BP and $30 \text{ g} \cdot \text{dm}^{-3}$ honey instead of sucrose. Plant regeneration proceeded on MS + $0.05 \text{ mg} \cdot \text{dm}^{-3}$ KIN and (1) $30 \text{ g} \cdot \text{dm}^{-3}$ sucrose or (2) $30 \text{ g} \cdot \text{dm}^{-3}$ honey

Callus induction medium	No. of explants	% of explants producing callus	Regeneration medium			
			with sucrose		with honey	
			No. of calli put on medium	No. of regenerants	No. of calli put on medium	No. of regenerants
MS – control	1114	68.85	409	193	416	194
MS + BP	891	69.70	326	145	336	212**
MS + honey	969	63.46	325	109**	327	160
MS + BP + honey	1259	77.36**	508	286	492	358***

Significant differences in frequency versus control (χ^2 test) are asterisked: ** – $p < 0.01$; *** – $p < 0.001$.

and plant regeneration was not determined by the osmotic potential of the media supplemented with BP and/or honey, but rather by the kinds of components present in banana fruit and honey.

Our general finding is that MS medium containing BP and honey instead of sucrose was the best medium for callus induction of *Miscanthus*, and regeneration efficiency for those calli was highest on MS with KIN and honey.

DISCUSSION

There is not much literature data on initiation of tissue culture of *Miscanthus × giganteus*, and even less information about *Miscanthus* tissue browning. Extensive experiments on *Miscanthus* callus induced from immature inflorescences and probes to inhibit phenolic production by the explants were described mainly by Lewandowski and Kahnt (1993). To reduce phenolic production they added to the callus induction medium such compounds as ascorbate, cysteine or activated charcoal, but did not achieve inhibition of tissue browning. Finally they obtained many embryogenic calli on rafts in liquid medium which removed phenolics from the tissue. In previous work we tried to inhibit phenolic production by immature inflorescences of *Miscanthus* by adding the PAL inhibitor AOA and also PVP to the MS medium, without positive results (Płażek et al., 2007). Other compounds such as chitosan or cysteine did not inhibit phenolic oxidation either, but media supplemented with these compounds yielded callus producing many ELS.

Tissue browning was less on MS medium containing honey instead of sucrose. This effect was probably due to specific organic compounds in honey which have antioxidant properties. Skrzypek and Dubert (2002) noted callus browning due to the high accumulation of phenolics in tissue culture of

field bean (*Vicia faba* var. *minor*). They showed that excess phenolic compounds inhibit the growth and development of callus but that a certain concentration of them is required for plant regeneration. A small amount of antioxidants added to the medium improved callus regeneration, probably via maintenance of an optimal phenolic pool and protection against oxidation.

In the first experiment of our study the number of explants inducing callus was highest on the control medium and the medium supplemented with chitosan. Apart from its ability to inhibit polyphenol oxidase, chitosan is suggested to be a signal molecule which initiates various anti-stress processes (Wojtaszek, 1997; Vander et al., 1998). Cysteine, BP or honey significantly decreased callus production as compared to the control medium, but in the second experiment the number of callusing explants on the media with BP + honey was similar to that on the control medium. Media with BP had higher osmolality than the control medium and the medium with honey. Since the efficiency of callus production was highest on MS supplemented with BP and honey, apparently it could not have been an effect of osmotic potential. Banana pulp stimulated root development already on callus-initiating medium, and it also increased the efficiency of plant regeneration. This is in accord with results given by Seeni and Latha (2000) who regenerated *Vanda coerulea* protocorms on medium supplemented with $35 \text{ g} \cdot \text{dm}^{-3}$ BP. Chugh et al. (2009) reported a positive effect of BP on root development in many orchid tissue cultures.

In the first experiment, regenerants were obtained only when the calli were induced on MS with BP. The twofold transfer of calli onto fresh medium may have been the problem there. In the first experiment, *Miscanthus* calli regenerated whole plants on MS with KIN, but regeneration remained at the leaf bud stage on MS supplemented with BAP.

This result is the opposite of what Lewandowski and Kahnt (1993) obtained; they regenerated whole plants of *Miscanthus* on MS with BAP. This effect probably is not due to the type of cytokinin but rather the concentration; in their work the KIN concentration ($0.05 \text{ mg} \cdot \text{dm}^{-3}$) in the plant regeneration medium was lower than the BAP concentration ($0.2 \text{ mg} \cdot \text{dm}^{-3}$).

In the second experiment the synergistic effect of BP and honey on the callus induction and regeneration process became evident. Plant regeneration did not differ between calli obtained from different MS media with KIN and sucrose. MS medium with KIN and honey instead of sucrose was the best regeneration medium for calli induced on MS with BP and honey or MS with honey only. When calli were induced on medium containing sucrose, their regeneration after transfer to medium with honey was not so efficient as on medium with sucrose. This reaction of the plant tissue to the change of carbon source can be explained by the presence of sugar-specific receptors or transporters. Sucrose in the medium is partly hydrolyzed to fructose and glucose during autoclaving, while honey contains mainly monosaccharides (fructose and glucose), which are transferred across cell membranes via specific transporters other than those for sucrose. This suggests that the carbon source should remain unchanged throughout tissue culture from callus initiation through the regeneration process.

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