THE ROLE OF OSMOTIC STRESS
DURING in vitro REGENERATION OF Triticum aestivum L.
AND Vicia faba ssp. minor 1*

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Introduction

Developmental processes during in vitro culture are influenced by many factors such as the plant genotype and explant type, the composition of the culture medium including the amino acids, carbohydrates and growth regulators, or water stress treatment. Changes in some physico-chemical conditions are required to start regeneration programs encoded in the genome. Carbon source in the regeneration induction medium affected the efficiency of that process. In most cases, sucrose as a major carbohydrate synthesized by plants or its constituent monosaccharides, glucose and fructose, were used in culture media as the carbon source to support cell growth and differentiation. However, several reports indicated that sucrose may not be the most suitable carbohydrate for the induction of somatic embryogenesis and plant regeneration in cell cultures [Strickland et al. 1987; Chu et al. 1990; Navarro-Alvarez et al. 1994; Blanc et al. 1999, 2002]. There are data confirming the important role of sugar content and osmotic potential of medium for somatic embryo formation. A high osmotic potential of medium efficiently induces cucumber somatic embryogenesis [Lou, Kako 1995]. Furthermore, it was suggested that osmotic stress induced by a high concentration of sugars started somatic embryogenesis in carrot [Kamada et al. 1989]. Osmotic treatment with a high concentration of sucrose was found to enhance the single-cell embryo formation in ginseng [Choi, Soh 1997]. Some reports [Jain et al. 1996, 1997; Iantcheva et al. 2005; Li et al. 2005] showed that not only media with elevated concentrations of carbohydrates, but also media containing additional carbohydrates, such as mannitol, stimulate plant differentiation in cultured cells. Carbohydrates, as regulators of osmotic potential of medium during in vitro cultures could modify the regeneration process. Shen et al. [1997] suggest that the exogenously applied mannitol can additionally act as free radical-scavenger and reduce oxidative damage of cells caused by hydroxyl radicals.

This work reports the effects of oxidative stress caused by osmotic potential of medium on the in vitro culture regeneration. Changes in the activity of antioxidative

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enzymes and endogenous level of hydrogen peroxide and plant regeneration frequency in response to the osmotic stress created by higher concentrations of sucrose and mannitol in shoot regeneration media were measured.

Material and methods

Experiments were carried out on callus of wheat (Triticum aestivum L.) cv. Kamila and field bean (Vicia faba ssp. minor) cv. Nadwińska Immature and mature embryos of wheat were cultured on MS medium [MURASHIGE, Skoog 1962] with 2 mg dm\(^{-3}\) 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g dm\(^{-3}\) sucrose, and 6 g dm\(^{-3}\) agar (pH 5.8), whereas field bean on MS medium containing 1 mg dm\(^{-3}\) casein hydrolysate, 750 mg dm\(^{-3}\) myo-inositol, 1 mg dm\(^{-3}\) 6-benzyl-aminopurine (BAP), 0.5 mg dm\(^{-3}\) naphthalene acetic acid (NAA), 0.25 mg dm\(^{-3}\) gibberellic acid (GA\(_3\)), 30 g dm\(^{-3}\) sucrose, and 6 g dm\(^{-3}\) agar (pH 5.8). Callus initiated from immature embryos, unlike that from mature embryos, was capable of shoot regeneration.

Four-week-old callus was placed for three weeks on media which modify callus differentiation: 3% sucrose (ok. -0.42 MPA), 6% sucrose (ok. -0.71 MPA) and 3% sucrose together with 7% mannitol (ok. -1.33 MPA). Material for analyses was collected 3 h and 1, 2, 4, 7, and 14 days after subculture. For the determination of regeneration potential calluses were transferred on to MS media without growth regulators.

The total \(\text{H}_2\text{O}_2\) content was measured according to Ishikawa et al. [1993], after callus homogenization with 5% trichloroacetic acid (TCA). For enzymes analysis calluses were homogenized with 1% PVP400 and 0.05 mol dm\(^{-3}\) phosphate buffer (pH 7.0) containing 0.1 mmol dm\(^{-3}\) ethylenediaminetetraacetic acid (EDTA) at 4°C. Total superoxide dismutase (SOD, EC 1.15.1.1.) activity was assayed according to the modified method of McCord and Fridovich [1969]. Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi [1984] and total peroxidases (POD) to Luck [1962]. For SOD isoenzyme analysis, tissue samples were homogenized in 0.05 M phosphate buffer (pH 6.8) containing 0.1 mmol dm\(^{-3}\) EDTA, 0.1% N,N,N',N'-tetramethylenediamine (TEMED), 1 mmol dm\(^{-3}\) dithiothreitol (DTT), and 0.1% ascorbic acid. SOD isozymes were separated by native PAGE using 11.5% polyacrylamide gels and 0.1 mol dm\(^{-3}\) Tris-glycine as an electrode buffer. Gels were stained by the method of Beauchamp and Fridovich [1971]. Proteins were assayed by Bradford’s dye-binding technique [1976] with bovine serum albumin as a protein standard.

Results and discussion

Little information is available regarding the effect of carbohydrates on in vitro embryogenesis and plant regeneration. Carbohydrates, besides their role as a carbon source, are known to function as osmotic regulators in culture media and this function was found to be critical for regeneration. Several reports showed that osmotic stress induced by carbohydrates, when used at elevated concentrations or by additional carbohydrates, such as mannitol, stimulate plant differentiation in cultured cells [Lou, Kak 1995; Jain et al. 1996; Choi, Soh 1997; Jain et al. 1997; Blanc et al. 1999, 2002; Li et al. 2005]. Mannitol acts mainly as osmotic regulators and, unlike sucrose, neither supports in vitro callus growth nor is metabolized in higher plants.

In our experiment the regeneration of shoots was observed only in callus
originated from immature embryos of both species. Regeneration efficiency depended on osmotic potential of the medium. After tree weeks culture on medium with 3% sucrose (control condition) there was no meristematic centers on the callus surface (Fig. 1), however further subculture on medium without growth regulators induced shoot formation. High concentration of sucrose in medium (6%) decreased the callus growth and blocked the regeneration even after passage on the regeneration medium. BLANC et al. [2002] suggested the participation of carbohydrates in synthesis and accumulation of starch reserves in callus cells, which were followed by the differentiation process: elongation and vacuolation. These processes could result in blocking regeneration potential in our culture, because the maintenance of small cell volumes is believed to be one of the factors needed to enable the onset of regeneration and to represent a dedifferentiated cell state with the potency to initiate a new developmental program [PASTERNAK et al. 2002].

In contrast, the presence of mannitol in medium caused the formation of meristematic centers and shoots on wheat callus much earlier as compared to other conditions. Shoots appeared on that medium one week before transfer onto regeneration media (Fig. 1). Moreover, regeneration efficiency was greatly enhanced. Wheat callus grown on medium with 7% mannitol regenerate approximately 50% more shoots as comparing with callus grown on medium without mannitol. Similarly to our experiment IANTCHEVA et al. [2005] reported that the osmotic pre-treatment had a primary effect of shortening the regeneration phase, and promoting the embryogenic potential of tissues.

However, field bean callus did not regenerate any shoots during sucrose and mannitol treatment. Shoots were observed 3 weeks later in regeneration conditions on calluses after 3% sucrose and 3% sucrose with 7% mannitol treatments. Therefore it is not clear how osmotic stress or reduction in cell water content influences the process of plant regeneration. Our experiment suggested that osmotic stress did not act directly, but could be a trigger of mechanisms leading to changes in developmental program. WETHERELL [1984] suggested that the osmotic stress may disrupt the plasmodesmatal connections between the pre-embryonic cells, thus making the cells physiologically isolated and allowing a greater number of cells to differentiate.

Changes in physico-chemical conditions of in vitro cultures could induce oxidative stress in cells. In our experiment, modification of medium osmotic potential and sugar composition (6% sucrose and 3% sucrose together with 7% mannitol) caused changes in endogenous H$_2$O$_2$ content and response of antioxidative enzymes system. Regenerating callus of wheat showed a lower content of H$_2$O$_2$ in all conditions in comparison to non-regenerating one and a higher activity of SOD, CAT and POD (Fig. 2).

Wheat callus; Kalus pszenicy

### Fig. 1.
Regeneration of wheat and field bean callus after three weeks on the medium with 3% sucrose (3% S), 6% sucrose (6% S) and 3% sucrose with 7% mannitol (3% S + 7% M)

### Rys. 1.
Regeneracja kalusa pszenicy i bobiku po trzech tygodniach wzrostu na pożywkach z 3% sacharozą (3% S), 6% sacharozą (6% S) oraz z 3% sacharozą i 7% manniotolem (3% S + 7% M)

Field bean callus; Kalus bobiku

### Fig. 2.
Regeneracja kalusa bobiku po trzech tygodniach wzrostu na pożywkach z 3% sacharozą (3% S), 6% sacharozą (6% S) oraz z 3% sacharozą i 7% manniotolem (3% S + 7% M)
Fig. 2. Endogenous amount of H$_2$O$_2$ and activity of SOD, CAT and POD in regenerable (R) and non-regenerable (NR) callus of wheat and field bean after 3 week growth on the medium with 3% sucrose (3% S), 6% sucrose (6% S) and 3% sucrose with 7% mannitol (3% S + 7% M). Means of three replicates ± SE
It suggests that the antioxidative enzymes effectively scavenge reactive oxygen species induced by high concentration of carbohydrates in medium. Endogenous H$_2$O$_2$ content showed a decrease in the early stages of wheat callus regeneration and then increase after 7th day. The maximum of SOD and CAT activities in wheat callus were observed on 7th day, then on 14th day of culture the SOD activity decreased. We suggest that a high production of endogenous H$_2$O$_2$ on 7th day, despite of high efficiency of enzymes, might act as a signal for initiation of regeneration mechanisms, because from that time the development of shoots was observed. POD activity was almost at the same level during whole wheat culture, both regenerating and non-regenerating, however higher POD activity was detected in regenerating callus in 14 days after treatment. Similarly to Kršník-Rasol [1991] report indicating peroxidase as a developmental marker in plant tissue we could suggest the role of POD in regeneration processes.

Field bean callus, potentially able to regeneration, showed a higher concentration of endogenous H$_2$O$_2$ in comparison with non-regenerating one and with wheat callus. Endogenous H$_2$O$_2$ content greatly increased from 7th day of culture. Enzymes activities in field bean were different from activities in wheat callus, too. The activity of SOD in field bean was almost ten times lower than in wheat. The highest values were detected on 14th day of culture. The fluctuations of CAT activity were observed during 14 days of culture. POD activity in field bean was higher than in wheat and decreased gradually during the subsequent days of culture. These data suggest that in field bean cells the mechanisms involved in antioxidative defense against free radicals generated during osmotic stress were different. Field bean calluses easily turned brown during culture as a result of polyphenol oxidation caused by peroxidases. Carbohydrates additionally might modulate polyphenol accumulation in cell culture [BLANC et al. 2002]. These processes generate H$_2$O$_2$ and free radicals. Probably too high level of reactive oxygen species could not be efficiently reduced by enzymes and results in tissue darkening and inhibition of field bean regeneration.
Gel electrophoresis showed one Mn and two Cu/Zn isoforms of SOD in wheat and field bean callus (Fig. 3). Activities of SOD increased together with the increase of osmotic potential of medium, however the most sensitive in wheat callus was the cytosolic isoform Cu/ZnSOD 1 (bound with lower mobility), whereas in field bean mitochondrial isoform MnSOD. These data revealed different mechanism and acting place of antioxidative enzymes.

Conclusions

The results of the experiment indicated that the oxidative stress and the osmotic potential play an important role in the regulation of regeneration processes. The mechanism involved in antioxidative defense during in vitro culture and the place of free radicals scavenging could determine the regeneration potential of cells. We suggest the function of SOD, CAT and POD as an indicator of tissue potential for regeneration. Activities of these enzymes and level of H$_2$O$_2$ can also be used in the prognosis of the ability of tissues to differentiate and regenerate.

References


THE ROLE OF OSMOTIC STRESS DURING in vitro culture ...


Key words: osmotic potential, oxidative stress, regeneration, Triticum aestivum L., Vicia faba L. minor

Summary

Developmental processes during in vitro culture are influenced by many environmental factors, which are required to start regeneration programs encoded in the genome. Isolation of explants and their in vitro culture might cause oxidative imbalance in cells. The aim of the experiment was to determine the effects of osmotic potential of medium on the in vitro regeneration focusing on the oxidative stress. Regenerating callus of Triticum aestivum and Vicia faba ssp. minor were obtained from immature embryos and non-regenerating from mature embryos. Callus after four week induction was placed for three weeks on the media with 3% sucrose (ok. -0.42 MPa), 6% sucrose (ok. -0.71 MPa), and 3% sucrose together with 7% mannitol (ok. -1.33 MPa). After that, the amount of endogenous H₂O₂ and activity of SOD, CAT and POD were measured during the subsequent days of callus culture. 6% sucrose inhibited regeneration of wheat callus, and addition of 7% mannitol stimulated shoot regeneration.
in comparison with 3% sucrose, whereas did not change regeneration potential of field bean callus. Regenerating callus of wheat accumulated less endogenous H$_2$O$_2$ than non-regenerating one and showed a higher activity of SOD, CAT and POD. Regenerating callus of field bean showed a higher amount of endogenous H$_2$O$_2$ and antioxidative enzymes. The results of the experiments indicated that the oxidative stress caused by changes of osmotic potential of medium and type of carbohydrates play an important role in regulation of regeneration processes. The activities of antioxidative enzymes and endogenous level of H$_2$O$_2$ can be used in prognosis for the ability of tissues to differentiate and regenerate.

ROLA STRESU OSMOTYCZNEGO PODCZAS REGENERACJI in vitro Triticum aestivum L. I Vicia faba ssp. minor

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Słowa kluczowe: potencjał osmotyczny, regeneracja, stres oksydacyjny, Triticum aestivum L., Vicia faba L. ssp. minor

Streszczenie

Na procesy rozwijające w kulturach in vitro wpływa wiele czynników środowiskowych, które mogą indukować proces regeneracji zakodowanego w genomie. Izolacja eksplantatów i indukcja kalusa w warunkach in vitro może powodować zmienny potencjał oksydo-redukcyjnego komórk. Celem eksperymentu było określenie wpływu potencjału osmotycznego pożywki na regenerację w kulturach in vitro z uwzględnieniem stresu oksydacyjnego. Regenerujący kalus Triticum aestivum i Vicia faba ssp. minor uzyskano z niedojrzałych zarodków, a niregenerujący z dojrzałych zarodków. Kalus po 4-tygodniowej indukcji rósł przez trzy tygodnie na pożywkach z 3% sacharozą (ok. -0,42 MPA), 6% sacharozą (ok. -0,71 MPA) i 3% sacharozą z dodatkiem 7% mannitolu (ok. -1,33 MPA). Po tym czasie, ilość endogennego H$_2$O$_2$ i aktywność SOD, CAT i POD była mierzona w kolejnych dniach regeneracji kalusa. 6% sacharozą hamowała regenerację kalusa pszenicy, a 7% mannitolu stymułował regenerację pędów w porównaniu z 3% sacharozą, ale nie zmieniał zdolności regeneracyjnych kalusa bobiku. Regenerujący kalus pszenicy akumuluł mniej endogennego H$_2$O$_2$ niż niregenerujący i wykazywał większą aktywność SOD, CAT i POD. Regenerujący kalus bobiku akumuluł większą ilość H$_2$O$_2$ i wykazywał wyższą aktywność enzymów antyoksydacyjnych. Wyniki eksperymentu wykazują, że stres oksydacyjny indukowany przez zmiany potencjału osmotycznego pożywki oraz rodzaj zastosowanych węglowodanów odgrywają rolę w regulacji procesu regeneracji. Aktywności enzymów antyoksydacyjnych i endogenous poziom H$_2$O$_2$ mogą być stosowane do prognozowania zdolności regeneracyjnych tkanek.

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