

**ARTICLE**

# Auxin autotrophic tobacco calli with modified aldehyde oxidase isoenzyme activities show enhanced abiotic stress tolerance

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**ABSTRACT** The growth, abiotic stress resistance and aldehyde oxidase (AO) activities of auxin autotrophic and heterotrophic tobacco calli were compared. The auxin-independent autotrophic calli maintained their growth rate even at 35°C temperatures, showed enhanced abiotic stress resistance in the presence of 50-300 mM NaCl, 0.1-5 mM KNO<sub>2</sub> or 0.1-10 mM H<sub>2</sub>O<sub>2</sub>. 0.1-5 mM H<sub>2</sub>O<sub>2</sub> caused a higher increase in glutathione S-transferase (GST) and glutathione peroxidase (GPÖX) activities of heterotrophic calli, however these enzymes worked in an elevated level in autotrophic lines under control circumstances and were induced differently under oxidative stress, indicating an altered signalling mechanism. AO activity could be detected by activity staining after native PAGE with indole-3-acetaldehyde (IAAId) substrate in both calli, which means that the enzyme catalyzing the last step in IAA biosynthesis is present in both tissues. Contrary to heterotrophic calli, in the auxin autotrophic cultures an isoenzyme with low mobility (A01) was detectable. 100 mM NaCl enhanced the A01 activity and a new isoenzyme (A02) was observed. The increase of the activities of these isoenzymes were higher in the autotrophic lines suggesting that the enhanced IAA biosynthesis can play a role in the recovery of growth under stress conditions.

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**KEY WORDS**

auxin heterotrophic and autotrophic calli, aldehyde oxidase, glutathione S-transferase and peroxidase, oxidative stress resistance

The requirement for auxin and cytokinin to maintain the growth of plant tissue cultures has long been known. Under certain conditions, however, proliferation can take place on medium containing no exogenous auxin and/or cytokinin; these hormone-independent cultures are referred to as autotrophic or habituated tissues, the process - in which the cells regain the hormone synthesizing capacity - is the habituation. Habituation defined as an epigenetic change, the sequence of DNA is not altered, but the DNA methylation patterns and expression of genes, posttranslational modifications can be different. It is extremely stable and heritable at cellular level.

Several processes can lead to an elevated auxin level, such as the enhanced or altered biosynthesis of indoleacetic acid (IAA; Jackson and Lyndon 1990; Szabó et al. 1994; Michalchuk and Druart 1999), or the different levels of IAA conjugation or degradation (Bouchet et al. 1978; Syono 1979; Michalchuk and Druart 1999).

Plants usually synthesize IAA from L-tryptophan, but there is evidence for tryptophan-independent IAA production (Crozier et al. 2000). The first enzymatic step of the major tryptophan-dependent pathway is catalyzed by aminotransfer-

ases with broad substrate specificities (Wightman and Forest 1978; Koshiba et al. 1993). The produced indole-3-pyruvic acid is extremely unstable and can be easily decarboxylated enzymatically or non-enzymatically to indole-3-acetaldehyde (IAAId). The last step is the conversion of IAAId to IAA by indole-3-acetaldehyde oxidase enzyme, this enzyme belongs to the aldehyde oxidase (AO) multigene family.

In plants, different isoforms of AO enzymes have been identified which have a rather broad substrate specificity and show organ specific distribution. AO enzymes can also oxidize indole-3-aldehyde (IAId) to indole-3-carboxylic acid in the peroxidative decarboxylation pathway of IAA causing an irreversible loss of IAA. AO oxidizes abscisic aldehyde to abscisic acid (ABA) either. Since members of the AO enzyme family catalyze the final step in the biosynthesis of phytohormones, plant AOs may have an important role in plant development and adaptation to environmental stresses (Koshiba et al. 1996; Sagi et al. 1998; Zdunek-Zastocka et al. 2004).

Glutathione S-transferases (GSTs) with high affinity for auxins and cytokinin have also been suggested to contribute to hormone homeostasis and responses to different stress factors, because this enzymes not only catalyze conjugation of reduced glutathione (GSH) with natural products and xenobiotics, but they function in the homeostasis of hormones and

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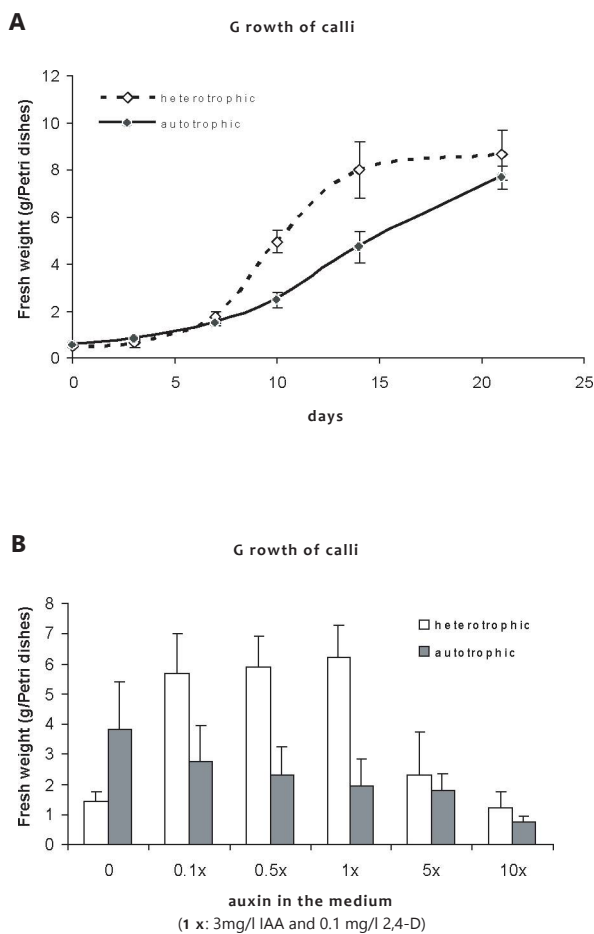


Figure 1. A: Growth of calli on RM media containing 17  $\mu$ M IAA and 0.5  $\mu$ M 2,4-D (in the case of auxin heterotrophic calli) or without exogenous auxins (for autotrophic calli). B: Growth of the auxin heterotrophic ( $\square$ ) and autotrophic ( $\blacksquare$ ) calli on 0.1-10 times external auxin containing RM media after 2 weeks. The amount of auxins for growing heterotrophic lines usually is 17  $\mu$ M IAA and 0.5  $\mu$ M 2,4-D (1x).

catalyze alternative GSH-dependent biotransformation reactions (Marrs 1996; Edwards et al. 2000, Basanti and Srivastava 2007). It was reported previously that the auxin autotrophic tobacco calli can be characterized with an elevated antioxidant activities and GSTs and glutathione peroxidases (GPOX) play an important role in their tolerance to 100 mM NaCl (Csiszár et al. 2004). In this paper we compared the growth and AO activities of auxin heterotrophic and autotrophic tobacco callus cultures under different conditions and evaluated the effect of exogenous  $H_2O_2$  on the two types of calli.

## Materials and Methods

### Plant material

The callus cultures originated from protoplasts of *Nicotiana tabacum* SRI plants (Csiszár et al. 2001). The auxin-require-

ing cultures were grown on a solid MS medium (Murashige and Skoog 1962) containing 2  $\mu$ M kinetin, 17.5  $\mu$ M IAA and 0.45  $\mu$ M 2,4-D, and the auxin autotrophic cultures were transferred onto the same medium without auxin. Each Petri dish contained 25 inocula; the weight of one inoculum was approximately 20 mg. The cultures were kept in a growth chamber at 25°C, under 8.4  $Wm^{-2}$  warm white fluorescent light (Tungsram F29 lamps, Hungary), and were analyzed in a 3-week period. Abiotic stress treatments were carried out by growing the calli in the growth chamber (Conviro) at 15-35°C, or by supplying the culture media with 50-300 mM NaCl or 0.1-5 mM  $KN_2O_2$ .  $H_2O_2$  was added aseptically in the final concentrations of 0.01-10 mM just before the medium became solid, and the inocula were plated immediately.

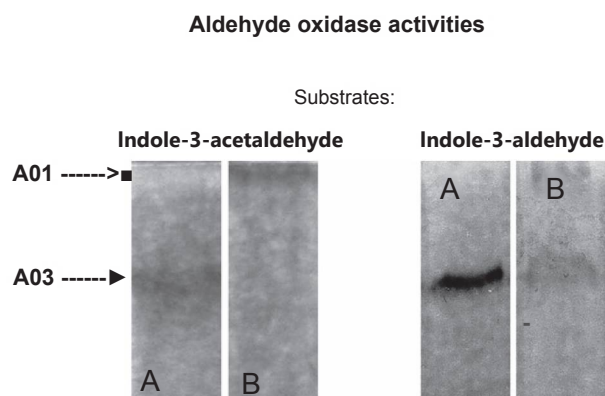
### Investigation of aldehyde oxidase in native gel

Two g of 2-week-old calli were homogenized with 2 ml of ice-cold extraction medium containing 250 mM Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), pH 8.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM reduced glutathione (GSH), and 2 mM dithiothreitol (DTT). The homogenized plant material was centrifuged at 10 000 g and 4°C for 25 min. The resulting supernatant was subjected to native polyacrylamide gel electrophoresis (PAGE) on 1.5-mm-thick slabs of 7.5% polyacrylamide gels in a Laemmli buffer system (Laemmli 1970) in the absence of SDS at 4°C. The gels were loaded with 100 mg protein. After electrophoresis, AO activity staining was developed at room temperature in a mixture containing 0.1 M TRIS-HCl, pH 7.5, 0.1 mM phenazine methosulphate, 1 mM MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide), and 1 mM indole-3-acetaldehyde (IAAld) or indole-3-aldehyde (IAld) substrate. AO activity was estimated on the basis of MTT reduction, which resulted in the development of specific formazan bands. The relative intensity of formazan bands was directly proportional to enzyme activity (Sagi et al. 1998). When IAAld was used as a substrate, the gel instantly became dark purple (Koshiha et al. 1996) however, we could definitely distinguish bands. Native PAGE was carried out with a Hoefer SE 600 (Amersham Pharmacia Biotech, USA).

### Activity measurements of glutathione S-transferase and peroxidase enzymes

The enzyme activities were determined 2 weeks after the transfer onto the MS medium. Two g of callus tissues was homogenized on ice in 4 ml extraction buffer (50 mM phosphate buffer pH 7.0, containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1% polyvinylpolypyrrolidone). The homogenate was filtered through two layers of cheese-cloth and centrifuged for 25 min at 15 000 g at 4°C. The supernatant was used for enzyme activity assays.

GST activity was determined spectrophotometrically



**Figure 2.** Aldehyde oxidase activities of 2-week-old tobacco calli with IAAld and IAld substrates after native-PAGE. A: auxin heterotrophic calli grown on RM medium containing 17 pM IAA and 0.5 pM 2,4-D; B: auxin autotrophic calli grown on RM medium without exogenous auxin.

by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB), according to Habig et al. (1974). Reactions were initiated by the addition of CDNB, and the increase in  $A_{340}$  was determined. One enzyme unit (U) is the amount of enzyme producing 1 pmol conjugated product in 1 min,  $e_{340} = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ .

Glutathione peroxidase (GPOX) activity was measured by the method of Awasthi et al. (1975), with cumene hydroperoxide as substrate. The reaction mixture contained 4 mM GSH, 0.2 mM NADPH, 0.05 U of glutathione reductase (GR, Type II from wheat, Sigma), 100 pi enzyme extract and 0.5 mM substrate in phosphate buffer (0.1 M, pH 7.0) in a total volume of 1 ml. The decrease of NADPH was followed by measuring the  $A_{340}$ ; the non-specific NADPH decrease was corrected for by using additional measurements without substrate.  $e_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ . One U is the amount of enzyme converting 1 pmol NADPH in 1 min. The protein contents of the extracts were determined by the method of Bradford (1976). The results are means  $\pm$ SD of three independent experiments.

## Results and Discussion

### Growth and AO activities of the auxin heterotrophic and autotrophic calli

The growth of the autotrophic cells exhibits a longer logarithmic, "rapid growth" phase: the fresh weight of the tissues growing on auxin-free medium were less than that of the heterotrophic calli growing in the presence of IAA and 2,4-D, but at the end of the 3rd week the difference disappeared (Fig 1A). The maximum growth of the heterotrophic calli was found in the presence of 17 pM IAA or 2.5 pM 2,4-D in the medium (Fig. 1B). Higher auxin concentrations proved supraoptimal. In the autotrophic tissues, all the used auxin concentrations inhibited the growth (Fig 1B). Their

sensitivity to the auxin content in the medium had been changed suggesting a possible difference in auxin-dependent gene regulation.

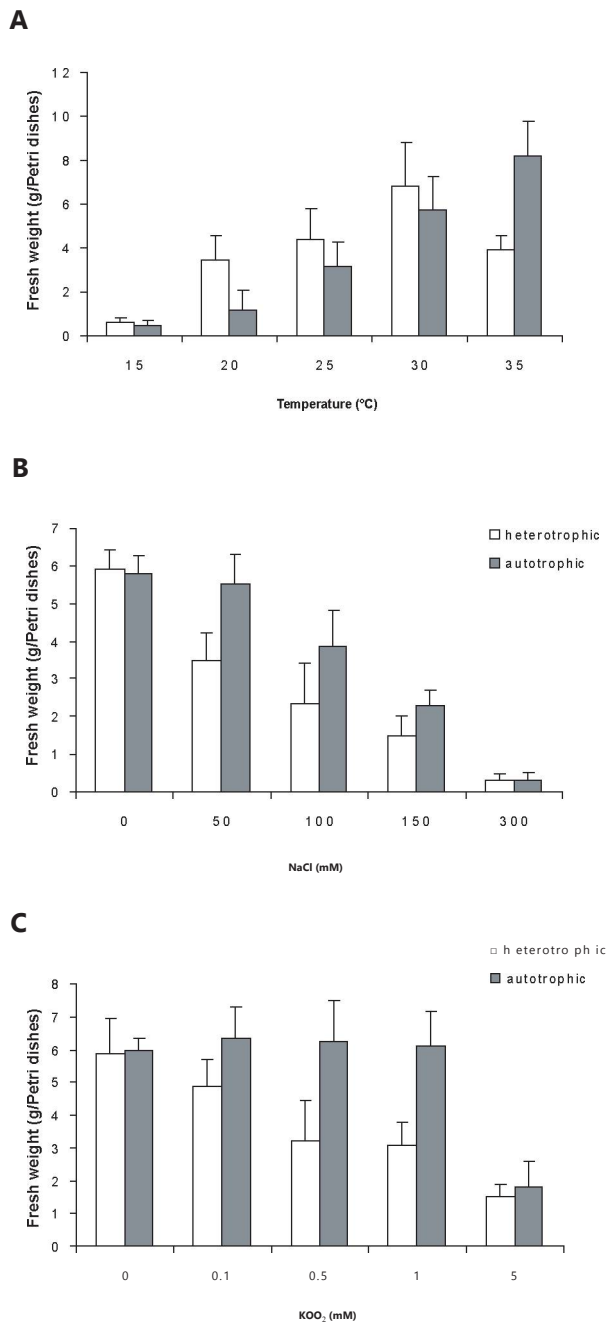
Investigation of the hormone metabolism in auxin autotrophic cultures revealed that their endogenous free auxin level can be higher than that in the heterotrophic lines (Nakajima et al. 1979). The major pathway for IAA biosynthesis appears to proceed via indole-3-acetaldehyde in plants, the final step is catalyzed by AO. We compared the isoenzyme pattern in the two types of calli by activity staining for AO after native PAGE.

AO activity could be detected both in the auxin heterotrophic and autotrophic calli using IAAld substrate, however the auxin autotrophic line contained a different AO isoenzyme (Fig. 2). Because this activity is present in the heterotrophic calli, our results demonstrate that the lack of the IAA synthesis of the cultured cells is not due to the limiting AO activity. However, in the autotrophic calli a new isoenzyme (AOI) was detected, which had considerable activity toward IAAld substrate.

Seo et al. (1998) reported that the auxin overproducing *Arabidopsis sur1* (*superroot1*) mutant had three AO isoenzymes (AOI-3). AOI had the lowest mobility, and its activity was significantly higher in mutant seedlings. The activity of this enzyme toward IAAld substrate was about 5 times higher in the extract of the *sur1* plants, supporting the possible role of AOI in IAA biosynthesis in *Arabidopsis* seedlings, but this isoenzyme showed also a relatively high activity with IAld substrate (Seo et al. 1998). Koshiba et al. (1996) found that maize AO had a high affinity for IAAld ( $K_m$  3-5 pM), indicating that even if a low concentration of IAAld was present in cells, the aldehyde could be converted into IAA. It is possible, that the newly activated enzyme, which is regulated presumably in a different way, in our auxin autotrophic calli is involved in the synthesis of IAA and in the auxin-independent growth of the autotrophic tissues. In our experiments the second isoenzyme (denoted by A03) exhibited a very strong staining with indole-3-aldehyde (IAld) substrate in the heterotrophic tissues, suggesting that in the IAA degradation pathway the oxidation of IAld catalysed by A03 has an important role (Fig. 2). This activity determinate the IAA levels not directly, because it is the last step of the peroxidases-initiated IAA inactivation pathway.

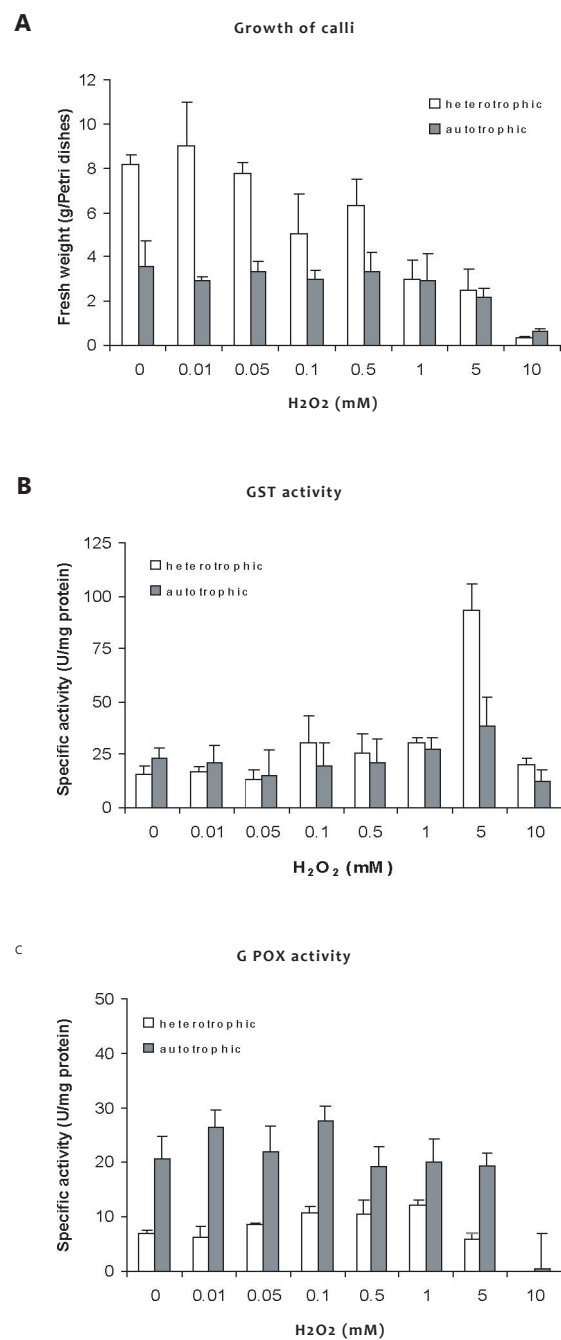
### The growth rate of the calli at different temperatures and in the presence of abiotic stressors

The effect of temperatures on the growth of calli were investigated on MS media used for maintaining the cultures; it contained 17 pM IAA and 0.5 pM 2,4-D for auxin heterotrophic calli, or no exogenous auxins in the case of autotrophic tissues. The growth rate of heterotrophic calli increased with increasing temperatures till 30°C, above this



**Figure 3.** Effect of different temperatures (A), 50-300 mM NaCl (B) and 0.1-5 mM KNO<sub>2</sub> (C) on the fresh weight of 2-week-old auxin heterotrophic and autotrophic calli. The used media are the same as in Fig. 1A.

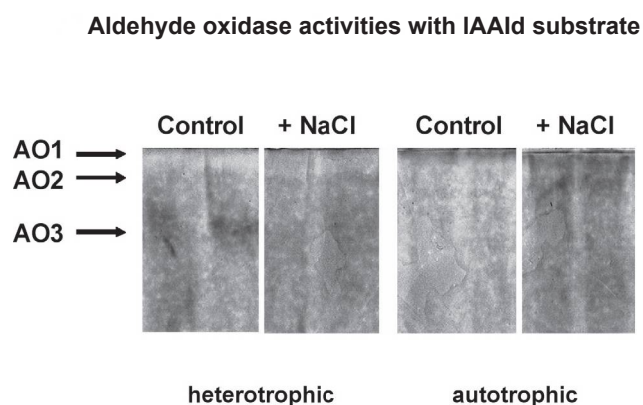
it was inhibited (Fig. 3A). Decrease in the growth rate may be an adaptation strategy for unfavourable circumstances; its role may be the reservation of the energy for defence mechanisms (May et al. 1998). The growth rate of auxin autotrophic calli showed an increasing tendency in our experiments; the fresh



**Figure 4.** Effect of exogenous H<sub>2</sub>O<sub>2</sub> on the growth (A), glutathione S-transferase (B) and glutathione peroxidase (C) activities of 2-week-old auxin heterotrophic and autotrophic calli.

weight of the 2-week-old calli at 35°C was even higher than that of the heterotrophic tissues. This indicates the enhanced stress tolerance of autotrophic tissues to high temperature.

Supplying the medium with 50-300 mM NaCl and 0.1-5 mM KNO<sub>2</sub> concentrations resulted in significant differences



**Figure 5.** Aldehyde oxidase activities of 2-week-old auxin heterotrophic and autotrophic calli grown on RM media supplied with 100 mM NaCl with IAAld substrate. RM media contained 17 pM IAA and 0.5 pM 2,4-D for the heterotrophic and no exogenous auxins for the autotrophic cultures.

in the growth of the two cultures and verified the enhanced abiotic stress resistance of the auxin autotrophic lines (Fig. 3B, C).

#### **The effect of exogenous H<sub>2</sub>O<sub>2</sub> on the growth, GST and GPOX activities of calli**

The common element of these different abiotic stressors is that they can, at the cellular level, enhance the generation of reactive oxygen species (ROS), such as superoxide radicals (O<sub>2</sub><sup>•-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radicals (OH<sup>•</sup>). It was suggested in our previous paper that the auxin autotrophic calli are more resistant to oxidative stress, and the enhanced GST and GPOX activities can play a role in this (Csiszár et al. 2004). We investigated further the tolerance of the two lines using exogenously applied H<sub>2</sub>O<sub>2</sub>.

The calli were transferred onto fresh MS medium containing 0.1-10 mM H<sub>2</sub>O<sub>2</sub>. The growth of 2-week-old auxin heterotrophic calli in the presence of 1 mM H<sub>2</sub>O<sub>2</sub> decreased by 60% comparing to the control (Fig. 4A); 10 mM H<sub>2</sub>O<sub>2</sub> caused the death of the tissues. The fresh weights of auxin autotrophic calli were ca. half of the heterotrophic ones, but their growth was inhibited only by the lethal 10 mM H<sub>2</sub>O<sub>2</sub>. This proves that the autotrophic calli really do possess a more effective H<sub>2</sub>O<sub>2</sub>-scavenging mechanism, but the concentration that activated the cell death at the two lines was the same.

Comparison of the GST activities in the heterotrophic and autotrophic calli revealed a considerable induction in the heterotrophic tissues, especially in the presence of 5 mM exogenous H<sub>2</sub>O<sub>2</sub>. The autotrophic callus had higher GST activity, but with the increasing H<sub>2</sub>O<sub>2</sub> concentrations this enzyme did not induce (Fig. 4B). Auxin autotrophic calli exhibited approximately two-fold higher GPOX activities than

the heterotrophic cultures, but the activation of this enzyme in the presence of H<sub>2</sub>O<sub>2</sub> was not so definite (Fig. 4C). It was established that auxin autotrophic tissues not only produce less ethylene than heterotrophic ones, but high external IAA and 2,4-D concentrations induced differentially their GST activities and the transferred bacterial mannopine synthase *masI'* promoter which, similarly to several GST genes, also contains stress- and hormone-inducible *e/v*-acting element (Marrs 1996; Guevara-Garcia et al. 1998; Csiszár et al. 2001). According to our former results, the crosstalk between the auxin- and ethylene-induced signal transduction pathways may differ in the two types of calli. The present results confirm the involvement of elevated GST and GPOX enzyme activities in oxidative stress resistance and the different induction of their activities in the presence of H<sub>2</sub>O<sub>2</sub> suggest an altered signalling mechanism in the auxin autotrophic lines.

#### **Effect of salt stress on AO isoenzyme activities of auxin heterotrophic and autotrophic tissues**

To investigate further the role of aldehyde oxidase activities in the abiotic stress tolerance of calli we supplied the media with 100 mM NaCl. Activity staining of samples originated from 2-week-old heterotrophic calli with IAAld substrate revealed that the activity of the AO3 isoenzyme decreased under salt stress, however the appearance of AO1 and even a new isoenzyme band, denoted by AO2, were detectable (Fig. 5). This new isoenzyme was also present in the salt-treated autotrophic tissues and its activity was higher than that in the stress-treated heterotrophic lines. In autotrophic calli the NaCl enhanced the activities both of AO1 and AO2 isoenzymes, indicating the increased IAA biosynthesis in stress conditions (Fig. 5).

Several studies investigated the effect of salinity on AO activities and regulation. NaCl usually increased the AO activity in roots of different plants, in some cases there was no effect in the leaves (Sagi et al. 1998; Omarov et al. 1998; Barabás et al. 2000; Tari et al. 2002). Using an ABA-deficient *Lycopersicon esculentum* mutant (*sitiens*) Dunlap and Binzel (1996) reported that NaCl reduced IAA levels in the roots of tomato plants independent of stress-induced ABA. In our experiments a new isoenzyme was detected in both type of calli and the higher induction was found in auxin autotrophic cultures. Our results suggest that the elevated or maintained IAA level and/or the altered signalling pathway in the auxin autotrophic calli may play a role in their enhanced stress tolerance.

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