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Original scientific paper

CHANGES OF ANTIOXIDATIVE ENZYMES IN *Impatiens walleriana* L. SHOOTS IN RESPONSE TO GENETIC TRANSFORMATION

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Impatiens walleriana L. shoots were inoculated with *Agrobacterium rhizogenes* A4M70GUS and the effects of genetic transformation on the catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX) activities in wounded region of stems and unwounded leaves were evaluated 10, 24, 240 and 720 hours after inoculation. Following *Agrobacterum* infection activities of plant antioxidative enzymes changed in a time-dependent manner indicating that dynamic processes occurred during plant-*Agrobacterium* interaction, plant cell transformation and formation of hairy roots. Appearance of hairy roots on wound sites of shoots was observed ten days after inoculation with *A. rhizogenes* and the root induction frequency was 100%. Among selected hairy root lines significant differences in growth rate and biomass production were observed and an average 3-fold increase in biomass production was observed for the best growing hairy root line compared with the untransformed roots. PCR analysis showed presence of *uid*A, *rol*B, *rol*C and *rol*D genes in all analyzed *I. walleriana* L. hairy root lines, while amplification fragment of *rol*A gene was detected in 83.3% transformed lines. Efficient transformation protocol for *I. walleriana* L described in this work offer possibilities to generate hairy root cultures for *in vitro* propagation of plant viruses.

Key words: Agrobacterium rhizogenes; antioxidative enzymes; *Impatiens walleriana* L.

Abbreviations: APX – ascorbate peroxidase; BSA **–** bovine serum albumin; CAT – catalase; *Cucumber mosaic virus* – CMV; DHAR – dehydroascorbate reductase; DNA – deoxyribonucleic acid; EDTA – ethylenediaminotetraacetate, GR – glutathione reductase; *Helenium virus S* – HSV; HR – hairy roots; *Impatiens necrotic spot virus* – INSV; LSD – least significant difference; MDAHAR – monodehydroascorbate reductase; NBT – nitro

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blue tetrazolium, PMSF – phenylmethylsulfonyl fluoride; POX – peroxidase; PVPP – polyvinylpolypyrrolidone; ROS – reactive oxygen species; SA – salicylic acid; SD – standard deviation; SOD – superoxide dismutases; TEMED – tetramethylethylenediamine; *Tobacco ringspot virus* – TRSV; *Tobacco streak virus* – TSV; *Tomato spotted wilt virus* – TSWV.

INTRODUCTION

Impatiens walleriana L. (Balsaminaceae) have been grown as horticultural plants both in Serbia and worldwide and have a significant role in improving public greeneries, gardens and balcones because of their attractive flowers and good branching (VUJOŠEVIĆ *et al.* 2008; MILOŠEVIĆ 2013). Numerous *Impatiens* cultivars with various flower colors and growth habits have been produced by conventional breeding methods. Different genetic transformation techniques have been established with the development of tissue culture approach and genetic engineering. The most popular used microorganisms are *Agrobacteruim rhizogenes* and *A. tumefaciens*, two Gram-negative soil bacterias. *A. rhizogenes*-mediated infection of the plant tissue cultures received attention 70 years ago as attempts were made to develop *in vitro* systems to study bacterial pathogenesis and hairy root (VENNA and TAYLOR 2007). Hairy root cultures have many advantages including biochemical and genetic stability, independence from seasonal and geographical conditions, rapid growth, and the ability to produce secondary metabolites at the levels comparable to those of the plants grown naturally. SHADWICK and DORAN (2007a, 2007b) and SHIT and DORAN (2009) demonstrated that hairy roots are a feasible means for *in vitro* propagation of plant viruses (*Tobacco mosaic virus* – TMV in *Nicotiana benthamiana* hairy roots). Like most ornamental plant species, the *Impatiens* cultivars are propagated vegetative which brings risks of transmitting viral diseases to the progeny and its further spread. *Impatiens* genus is highly susceptible to several viruses (*Tomato spotted wilt virus* – TSWV*, Impatiens necrotic spot virus* – INSV, *Tobacco streak virus* – TSV, *Tobacco ringspot virus* – TRSV, *Helenium virus S* – HSV and *Cucumber mosaic virus* – CMV (LEMBO DUARTE *et al*. 2007)) and virus infection is limiting factor in commercial production. *In vitro* culture techniques offer possibilities for virus elimination from *Impatiens* species (MILOŠEVIĆ *et al.* 2011, 2012). Since living cell is necessary for plant virus replication, hairy root culture can represent a suitable pool for virus preservation and availability when it is needed. Genetic transformation with *A. rhizogenes* has been extensively studied in diverse plant species. A search of the SCOPUS database using “hairy root” as a keyword query resulted in 2000 articles, the majority of which dated from the 1990s (ONO and TIAN 2011). The first report of *A. rhizogenes* mediated transformation of *Impatiens* (*I. hawkerii* Bull. – MILOŠEVIĆ *et al.* 2009) provided a transformation protocol and successful production of hairy roots.

DITT *et al*. (2001) show that a number of plant transcripts have their expression altered at 24 and 48 h after interaction with *Agrobacterium*, and that the proteins encoded by these genes have a putative role in plant signal transduction and in defense response. *Agrobacterium*-plant interactions often lead to increased level of the reactive oxygen species (ROS: superoxide radical [□O_2^-], hydrogen peroxide [H_2O_2]), hydroxyl radical [□OH], singlet oxygen [$^1O_2$]) in the plant cells. Oxidative stress can elicit positive responses such as normal cellular proliferation, activation of transcription factors or gene expression, as well as negative responses such as growth inhibition or cell death (DAN *et al*. 2009). To mitigate and repair damages triggered by oxidative stress, plants evolved a series of both enzymatic (including superoxide dismutase – SOD, catalase – CAT,

nonspecific peroxidase – POX, ascorbate peroxidase – APX, glutathione reductase – GR, monodehydroascorbate reductase – MDAHAR, dehydroascorbate reductase DHAR) as well as non-enzymatic (e.g. glutathione, ascorbate, tocohpherols) antioxidant defense mechanism. SODs (Fe-SOD, Mn-SOD and Cu/Zn-SOD) convert the superoxide radical to H_2O_2 and constitute the first line of defense against ROS. CAT, APX and other variety of POX catalyzes the decomposition of H_2O_2 and plays an important role in controlling the homeostasis of ROS. CAT is only active at relatively high H_2O_2 concentrations, while lower levels of H_2O_2 are eliminated by APX and other enzymes. POX metabolizes H_2O_2 to H_2O. Many dynamic changes in antioxidatve enzymes as a part of the ROS metabolism are still under discussion and there are contradictory data described by many authors (VAN BREUSEGEM *et al*. 2001; APEL and HIRT 2004; RADWAN *et al.* 2010).

Little is known about the oxidative response of plants during genetic transformation and the aim of this work is to elucidate level of oxidative stress in *I. walleriana* L caused by transformation with *A. rhizogenes* A4M70GUS. We have analysed the activity SOD, CAT and POX, and SOD isoforms pattern in wounded region of stems and in unwounded leaves following inoculation. In adition, we report an efficient method for the production of hairy roots, a convenient tool for *in vitro* propagation of plant viruses.

MATERIALS AND METHODS

Plant material

Micropropagated shoots of *I. walleriana* L.with three nodes were used for transformation with *A. rhizogenes* A4M70GUS. Inoculation was performed by puncturing internodes with a hypodermic needle, dipped into bacterial suspension (OD 600 nm = 0.98). The bacterial strain *A. rhizogenes* A4M70GUS was cultured on solid YEB medium at pH 7.2 supplemented with 100 mg l^{-1} neomycin. Bacterial suspension used for transformation was prepared by transferring a single bacterial colony in 10 ml liquid YEB medium. The inoculated shoots were cultured on MS medium (MURASHIGE and SKOOG 1962) containing 30 g l^{-1} sucrose, 100 mg myoinositol, 7 g l^{-1} agar in growth chamber at 25 ± 2 C°, with 16 h photoperiod at photon flux of 50 µmol s^{-1} m^{-2}. Developed hairy roots are excised from the shoot explants after four weeks and transferred onto MS medium with 200 mg l^{-1} cefotaxime (Tolycar, Jugoremedia, Zrenjanin, Serbia) and individual transformed root cultures are maintained by subculturing root tips every four weeks on the same medium.

Protein extraction

Plant material (wounded region of stems and unwounded leaves) were homogenized in liquid nitrogen, and crude extracts of soluble proteins were prepared with a buffer containing 50 mM Tris, 1 mM ethylenediaminetetraaceticacid (EDTA), 30% glicerol, 1.5% polyvinylpolypyrrolidone (PVPP), 10 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged (12 000 x g) for 5 min at 4 °C and the supernatants were filtered prior to protein determination according to BRADFORD (1976) with bovine serum albumin (BSA) as standard. The supernatants were kept at −70 °C until use for the assays.

SOD isoforms (25 µg proteins/lane) were separated according to BEAUCHAMP and FRIDOVICH (1971) by Native Page on 7% polyacrylamide gels at 120 V, using the vertical Serva

Blue Line system immersed in an ice bath, until the loading dye reached the end of a 10 cm long gel (≈3 h). SOD activity assay involved a 30 min-preincubation step with inhibitors that allow discrimination among different isoforms: Cu/Zn-SOD was inhibited by 1 mM KCN, both Cu/Zn-SOD and Fe-SOD were inhibited by 5 mM H_2O_2 while the control gels incubated in buffer (100 mM K-P pH 7.8) displayed all isoforms including Mn-SOD. The gels were stained in assay reagent (nitroblue tetrazolium chloride (NBT), riboflavin, 250 mM EDTA and tetramethylethylenediamine (TEMED) in potassium-sodium buffer) in darkness for 30 min, rinsed, and exposed to light until white bands were appeared on a purple background.

SOD activity was determined spectrophotometrically (Agilent 8453 spectrophotometer, Life Sciences, USA) according to BEYER and FRIDOVICH (1987). A reaction mixture (3 ml) contained of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 260 mM L-metionin, 1.5 mM NBT, 0.04 mM riboflavin, and 0-50 µl of crude protein extract. The mixture was kept under fluorescent light (Tesla Pančevo, 65 W) for 60 min and 25±2 °C. One unit of enzyme activity (U) was defined as the amount of enzyme where the NBT photochemical reduction (to blue formazan) ratio was 50%. NBT reduction rations were measured with ELISA microplate reader (adjusted to 540 nm). The mixtures without enzyme extract were used as a control.

CAT activity was determined spectrophotometrically by measuring the H_2O_2 absorbance decrease (µmol $min^{-1}mg^{-1}$) at 240 nm (AEBI 1984). Reaction mixtures (3 ml) contained 50 mM potassium-sodium phosphate buffer (pH 7.0), 20 µl of crude protein extract and 30 mM H_2O_2 (A_{240} ε=0.04 $mM^{-1}cm^{-1}$).

POX activity was assayed by measuring the increase in absorbance at 430 nm (KUKAVICA and VELJOVIĆ-JOVANOVIĆ, 2004). Reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 6.5), 10 µl of crude protein extract, 60 µl 1 M pyrogallol (Sigma, A_{430} ε=2.4 $mM^{-1}cm^{-1}$) as a hydrogen donor, and 30 µl 1 M H_2O_2.

PCR analysis

Genomic DNA was extracted from hairy roots frozen in liquid nitrogen by using CTAB DNA isolation method (ZHAO *et al*. 1994). DNA from nontransformed *I. walleriana* L. roots and plasmid DNA were used as negative and positive control, respectively. To confirm transformation DNA of all established lines were analyzed by PCR amplification using *uid*A, *rol*A, *rol*B, *rol*C and *rol*D primers (Table 1). To confirm the absence of *Agrobacterium* from the hairy root lines, PCR was carried out using primers specific for *vir*D1 gene (Table 1). PCR conditions for all analyzed genes were: initial denaturation at 95 °C for 5 min, followed by 38 cycles of melting at 95 °C for 1 min, anneling at 60 °C for 1 min, elongation at 72 °C for 2 min and final extension at 72 °C for 10 min. PCR products were visualized by electrophoresis separation (Serva Blue Marin 100) on 1.5% agarose gels in 1×TBE buffer and staining with ethidium bromide.

Growth parameters of hairy roots

For testing biomass accumulation, 150 mg of hairy root tips of 12 lines and control were subcultured in 50 ml of MS liquid medium lacking plant growth regulators and grown on a rotary shaker set (Adolf Kühner AG CH-4127, Birsfelden, Switzerland) at 60 rpm in dark at 25 ± 2 °C for a total period of 35 days.

Table 1. List of primers used for PCR analyses

Gene	Primer sequences 5' -3'	Size of amplified fragment (bp)
*uid*A	CCC GGC AAT AAC ATA CGG CGT G	366
	CCT GTA GAA ACC CCA ACC CGT G	
*vir*D1	ATG TCG CAA GGC AGT AAG	441
	CAA GGA GTC TTT CAG CAT G	
*rol*A	GTT AGG CGT GCA AAG GCC AAG	203
	TGC GTA TTA ATC CCG TAG GTC	
*rol*B	AAA GTC TGC TAT CAT CCT CCT ATG	348
	AAA GAA CGT GCA AGC TAC CTC TCT	
*rol*C	TAC GTA GAC TGC CCG ACG ATG ATG	342
	AAA CTT GCA CTC GCC ATG CCT CAC	
*rol*D	CCT TAC GAA TTC TCT TAG CGG CAC C	477
	GAG GTA CAC TGG ACT GAA TCT GCA C	

Statistical analyses

All data represent means of three independent experiments and were statistically analyzed using one-way ANOVA followed by Least Significant Difference (LSD) test at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Genetic transformation using *Agrobacterium* represents one type of biotic stress. It was documented that pathogen infection trigger oxidative burst characterized by rapid ROS production, leading to the activation of the plant defense (TORES *et al*. 2006). Thus, pathogenesis related accumulation of ROS can be expected after inoculation with *A. rhizogenes*, both in the site of infection and beyond initial infection site, as a part of local and systemic defense. During the stress induced by ROS plants produce CAT, APX, and other ROS-scavenging enzymes to protect their cells against new stresses and prevent ROS toxicity (GECHEV *et al*. 2006). These enzymes could restrict the ROS-induced host plant cell damage and finely tune ROS-dependent signal transduction (TORRES *et al*. 2006). As expected, the changes in antioxidative enzyme activities of *I. walleriana* L were found to depend of period after inoculation. The different activities of SOD, CAT and POX which were observed during genetic transformation suggests that wounded region of stems and unwounded leaves of *I. walleriana* L. have different dynamics or mechanisms of response to oxidative stress caused by *Agrobacterium* infection.

The maximum activity of SOD was detected 10 h in wounded region of stems and 240 h in unwounded leaves (Fig. 1). In wounded region of stems SOD activity was increased 10 h after transformation and then decreased, indicating the local defense response on the infection. In leaves its activity increased rapidly during the first 240 h, to levels 2-fold higher then control (Fig. 1) and this can be connected with systemic plant defense signaling. The activities of these enzymes then decreased rapidly (reaching 2-fold lesser than in control).

The isoenzyme profiles of SOD obtained from wounded region of stems and unwounded leaves are shown in Fig. 2. In all samples Mn-SOD, Fe-SOD and Cu/Zn-SOD isoforms were detected. Also, it was detected that transformation induced the appearance an extra Mn-SOD isoform in wounded region of stems 720 hours after inoculation. Existing evidence suggested that

activity of specific MnSOD form, MnSOD-2, was associated with process of rhizogenesis and root development (KONIECZNY *et al*. 2014). Thus, detected presence of MnSOD-2 in wounded region (but not in the leaves) 720 h after inoculation can be correlated with *I. walleriana* L hairy root appearance and growth.

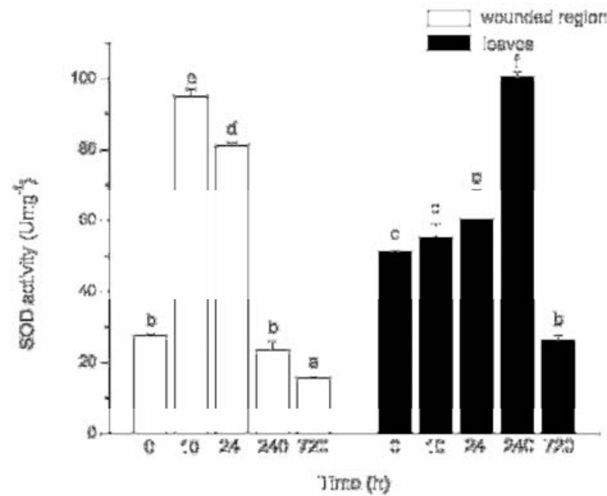

Fig 1.Total SOD activities in wounded region of stems and unwounded leaves of *I. walleriana* L. Each point represents the mean of three replicates ± SD, at a significance level of $P < 0.05$

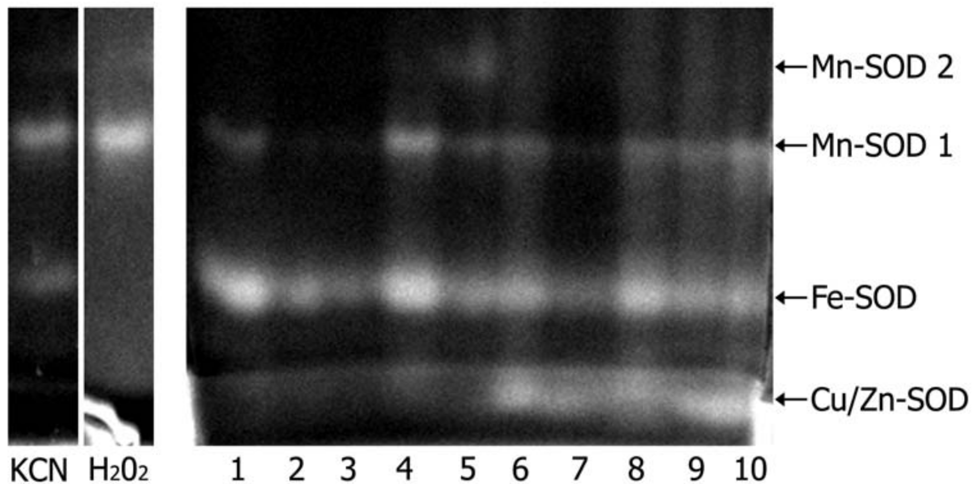

Fig 2. Separation of SOD isoforms from wounded region of stems (1– control, 2 – 10, 3 – 24, 4 – 240, 5 – 720 h) and unwounded leaves (6 – control, 7 –10, 8 – 24, 9 – 240, 10 – 720 h) of *I. walleriana* L.

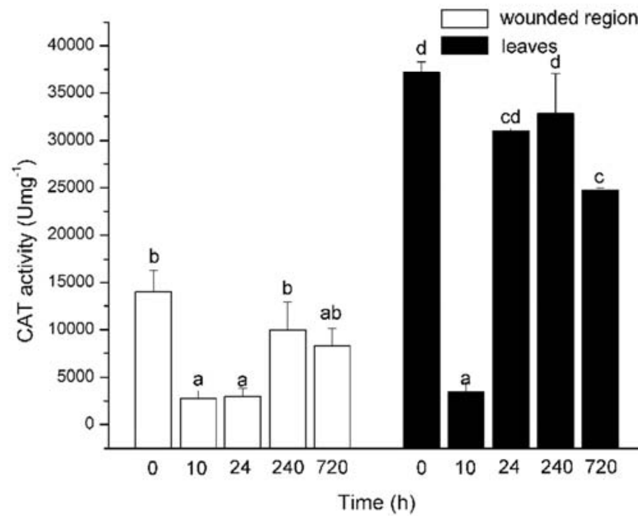

Fig 3.Total CAT activities in wounded region of stems and unwounded leaves of *I. walleriana* L. Each point represents the mean of three replicates ± SD, at a significance level of $P < 0.05$

Although different authors observed that POX production is stimulated when the plants are infected by pathogens (RIEDLE-BAUER, 2000; MILAVEC *et al*. 2001; RADWAN *et al*. 2010) transformation of *I. walleriana* L. with *A. rhizogenes* A4M70GUS has decreased production of POX 10 h after inoculation. POX level in wounded region of stems and unwounded leaves were about 10.4-fold and 19.6-fold lesser, respectively, compared to the uninfected plants (Fig. 4). At the same time point, level of CAT (Fig. 3) was also decreased (5-fold in wounded region of stems and 10.7-fold in leaves lesser then the control). It was known that in defense against infection, contrary to the SOD activities, POX and CAT activities can be inhibited as effect of salicylic acid (SA) signaling connected with pathogenesis-related responses (RADWAN *et al*. 2010). After that period improved production of POX activity was detected, and the highest level was obtained 720 h after transformation (Fig. 4). The significant increase of POX activities 240 and 720 h after inoculation with *A. rhizogenes* was probably correlated with formation of hairy roots, observed 10 days after inoculation, since POX activity has always been a marker for root induction (SAXENA *et al*. 2000; FU *et al*. 2011). Although the early stages of plant rhizogenesis coincided with low activities of the CAT and POX enzymes, postdetermination phase of rooting is characterized with high POX activities and this enzyme play role in organ growth and tissue maturation (KONIECZNY *et al*. 2014).

From the other side, early transcription of T-DNA genes can be detected 24 or 48 h upon infection (NARASIMHULU *et al*. 1996) and expression of *Agrobacterium* individual *rol* genes in plant cells has an effect on the metabolism of ROS (BULGAKOV *et al*. 2013). The combined action of the *rol* genes with other T-DNA oncogenes resulted in a moderate activation of the ROS-

detoxifying genes (SHKRYL *et al*. 2010) and of ROS-detoxifying enzymes in hairy roots (NIKRAVESH *et al*. 2011). Especially the *rolC* and *rolB* genes, acting as ROS suppressors in transformed cells, were able to stimulate production of antioxidative enzymes and prevent oxidative damage (BULGAKOV *et al*. 2012, 2013).

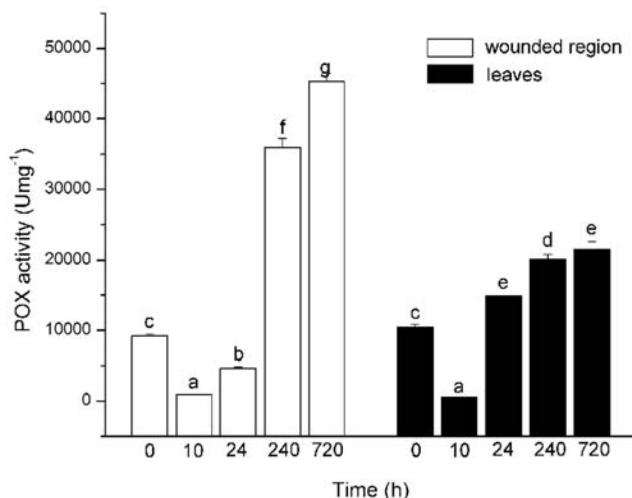

Fig 4.Total POX activities in wounded region of stems and unwounded leaves of *I. walleriana* L. Each point represents the mean of three replicates ± SD, at a significance level of $P < 0.05$

The initial appearance of hairy roots on wound sites of *I. walleriana* L. shoots was observed within ten days after inoculation with *A. rhizogenes* A4M70GUS. The root induction frequency was 100% and mean number of roots from inoculation sites was 9.05 (Fig. 5a, b). High efficiency of transformation with *A. rhizogenes* is common for *Impatiens* genus (MILOSEVIĆ *et al*. 2009), as well as for some other plant species (BERGIER *et al*. 2012).

Following the transfer to MS plant grow regulators-free medium 55 lines were established. Although in many cases the *rol* genes ensure a high growth rate of transformed cells and their hormonal independence, the hairy root lines of *I. walleriana* L. showed considerable phenotypic variations, particularly growth rate and roots morphology. These selected lines were grouped into five types, based on hairy root (HR) phenotypes and their growth dynamics (Fig 5d, e, f). HR lines of type I (two selected lines) and II (eight HR lines) had long primary roots with good lateral branching and vigorously growing. Among them, the line 32 had many root hairs (Fig 5e). Lines of type III (nineteen HR lines) were thin but good growing. Type IV (nineteen HR lines) had lower grow, while HR lines of type V (seven lines) were not grown on MS medium. Twelve axenic *I. walleriana* L. hairy root lines obtained through inoculation with *A. rhizogenes* A4M70GUS were selected for further investigation.

We have studied the biomass production of twelve hairy root lines *I. walleriana* L. as well as untransformed root cultures. HR line 32 (type II) grew more rapidly and branched more

frequently than all others HR lines (Fig. 5d, e, f) and this line showed the highest biomass production (41.60 gl^{-1}) of an average 3-fold increase compared with the control roots (Fig. 6). Contrary to the line 32, HR line 21 (type IV) exhibited the lowest growth rate and biomass production more similar to the untransformed control roots (Fig. 6). These differences in growth and morphology between HR lines is difficult to explain, largely because it is unclear to what extent the oncogene rearranges the genetic apparatus of cells and to what extent T-DNA integration site in the plant genome (or other factors) determine individual *rol* genes expression.

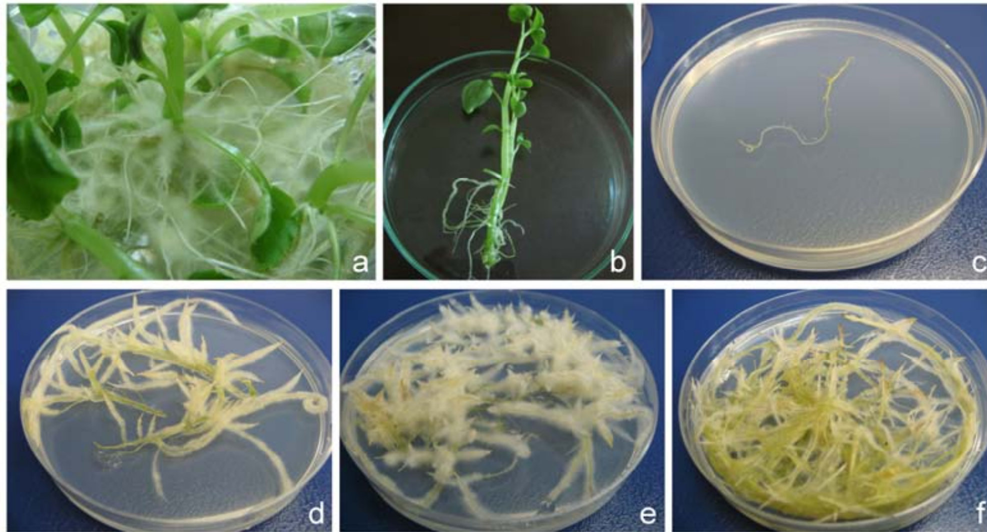

Fig.5 Introduction and establishment of hairy root from shoot of *I. walleriana* L. in response to *A. rhizogenes* A4M70GUS. a-b) *In vitro* shoots of *I. walleriana* with developed hairy roots, c) growth of culture of nontransformed roots, d) L24, e) L32, f) L48 HR lines

Screening of selected hairy roots lines was performed by PCR assay for *uid*A, *rol*A*, rol*B, *rol*C and *rol*D genes (Fig. 7). PCR showed that amplification products of 348 bp (*rol*B) or/and 342 bp (*rol*C) or/and 477 bp (*rol*D) were detected in all hairy roots lines and not detected in untransformed control roots. Ten of the twelve (83.3%) analyzed hairy root lines had positive amplification signal for 366 bp fragment of *uid*A gene indicating presence of this gene into host cell genome. High percent of *uid*A positive hairy root lines has been reported for some other species transformed with the *A. rhizogenes* A4M70GUS (NIKOLIĆ *et al*. 2003/4; ZDRAVKOVIĆ-KORAĆ *et al*. 2004; SRETENOVIĆ-RAJČIĆ *et al*. 2006; MILOŠEVIĆ *et al.* 2009; VINTERHALTER *et al*. 2011; TADIĆ *et al*. 2014). A distinct band of 441 bp corresponding to the *vir*D gene was not obtained in the DNA of all hairy root lines and control roots indicating absence of contamination with the *Agrobacterium*.

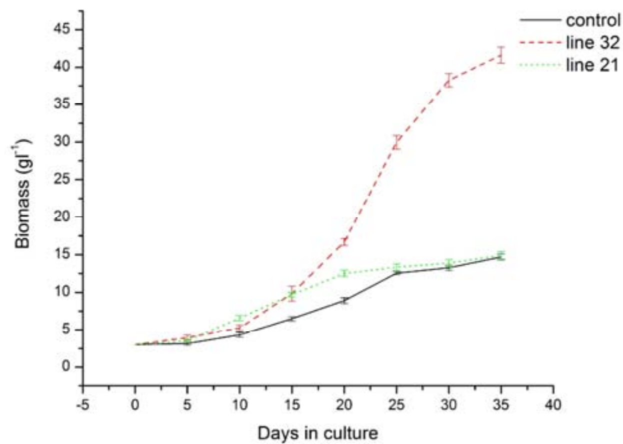

Fig 6.Time course of growth of *I. walleriana* L. hairy root lines L21 (the lowest growth rate) and L32 (the highest growth rate) and control untransformed roots. Each point represents the mean of three replicates ± SD, at a significance level of $P < 0.05$

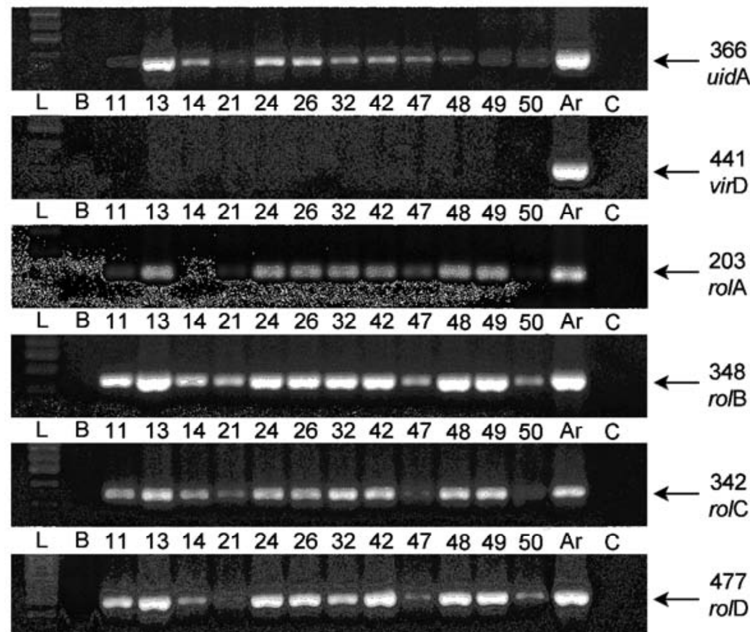

Fig 7.PCR analysis of genomic DNA isolated from hairy roots of *I. walleriana* L. Lanes: L – ladder, B – blank, 11-50 – hairy roots lines, C – untransformed control roots, Ar – positive control (pRiA4M70GUS)

In conclusion, it is evident that after *Agrobacterum* infection activities of plant antioxidative enzymes changed in a time-dependent manner, both in wounded and unwounded parts of *I. walleriana* L, showing that dynamic processes occurred during transformation. These results indicate that antioxidative enzymes plays important role during plant-*Agrobacterium* interactions, plant cell transformation and formation of hairy roots. In addition, the efficient transformation protocol achieved here offer possibilities to generate *I. walleriana* L. hairy roots for *in vitro* propagation of plant viruses.

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PROMENE ANTIOKSIDATIVNIH ENZIMA U IZDANCIMA *Impatiens walleriana* L. KAO ODGOVOR NA GENETIČKU TRANSFORMACIJU

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Izvod

Izdanci *Impatiens walleriana* L. su inokulisani sa *Agrobacterium rhizogenes* A4M70GUS i praćen je efekat genetičke transformacije na akivnost katalaza (CAT), superoksid dismutaza (SOD) i peroksidaza (POX) u regionu povrede izdanka kao i u nepovređenim listovima 10, 24, 240 i 720 sati nakon infekcije. Promene u aktivnosti antioksidativnih enzima biljke u zavisnosti od vremena nakon infekcije ukazuju na dinamične procese koji se odigravaju tokom interakcije biljke i Agrobakterije, transformacije biljnih ćelija i formiranja "hairy roots" (transformisanih korenova). Pojava transformisanih korenova je uočena 10 dana nakon inokulacije i učestalost njihove indukcije iznosila je 100%. Između izabranih "hairy roots" linija postojale su značajne razlike u stopi rasta i uvećanju biomase, a za transformisanu liniju korenova koja je pokazivala najbolji rast zabeležen je trostruko veći porast biomase u odnosu na netransformisane korenove. PCR analizom je potvrđeno prisustvo *uid*A, *rol*B, *rol*C and *rol*D gena u svim analiziranim linijama transformisanih korenova *I. walleriana* L. dok je fragment amplifikacije *rolA* gena detektovan kod 83.3% transformisanih linija. Efikasni protokol transformacije *I. walleriana* L koji je opisan u ovom radu pruža mogućnost stvaranja kultura transformisanih korenova u cilju *in vitro* propagacije biljnih virusa.

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