ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume LXI 141 Number 5, 2013
http://dx.doi.org/10.11118/actaun201361051279

RESOLVING BROWNING DURING THE ESTABLISHMENT OF EXPLANT CULTURES IN *VICIA FABA* L. FOR GENETIC TRANSFORMATION

Helena Klenotičová, Iva Smýkalová, Lenka Švábová, Miroslav Griga

Received: April 30, 2013

Abstract

KLENOTIČOVÁ HELENA, SMÝKALOVÁ IVA, ŠVÁBOVÁ LENKA, GRIGA MIROSLAV: *Resolving browning during the establishment of explant cultures in Vicia faba L. for genetic transformation.* Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 2013, LXI, No. 5, pp. 1279–1288

Optimisation of *in vitro* regeneration systems of two explant types for low-tannine cultivars of faba bean based on culturing of shoot apices and cotyledonary nodes were provided by usage of various antioxidants - ascorbic acid, citric acid, glutathione and activated charcoal. In subsequent testing, the combined effects of antioxidants with transformation co-cultivation compounds acetosyringone and L-cysteine was studied. The application of antioxidants lead to decreased callogenesis, citric acids treatments (50 $mg.l^{-1}$) dramatically decreased necrotic response of explants. However, citric acid, used together with ascorbic acid completely inhibited shoot growth in shoot apex cultures. Glutathion evoked hyperhydricity of explants. Activated charcoal induced rooting on media which are commonly used for shoot proliferation. Combination of acetosyringone with antioxidants influenced shoot proliferation, except of variant with ascorbic acid. Citric acid was the best and universal antioxidant in faba bean *in vitro* cultures and its use is recommended for faba bean genetic transformation experiments.

acetosyringone, antioxidants, citric acid, co-cultivation agents, faba bean, organogenesis

The efficient protocols for *in vitro* regeneration are necessary for the application of biotechnological methods, including transformation methodology into plant breeding.

While the establishment of *in vitro* culture in pea or soybean is relatively easy, faba bean represents one of *in vitro* recalcitrant legume due to frequent deterioration of explant material and cultivated tissues as a result of the action of phenolic compounds (Pickardt, 2003; Griga *et al.*, 1986, 1987; Griga, 1988). In faba bean, organogenic *in vitro* systems are the most frequently used, such as multiple shoot culture initiated from meristems of cotyledon explants with embryonal axis (Anwar *et al.*, 2011), and/or apices (Abdelwahd *et al.*, 2008; Fakhrai *et al.*, 1989; Griga, 1988; Griga and Klenotičová, 1994; Selva *et al.*, 1989; Tejklová *et al.*, 1984).

Transformation of plant genome using *Agrobacterium tumefaciens* is the exploitation of the process of pathogen infection (Dan, 2008). The defence strategy of legumes, starts with rapid accumulation of phenols at the infection site to slow growth rate of pathogen and activation of specific defence mechanisms consisting of synthesis molecules related to the stress (Mohamed *et al.*, 1992). Initial response of plants to pathogen attack is an oxidative burst with rapid and transient production of reactive oxygen species (ROS) (Wojtaszek, 1997 in Dan, 2008). In faba bean, even germination of seeds under dark conditions induce increased activity of antioxidant enzymes (Younis *et al.*, 2010) and further, faba bean cuttings of explants lead to rapid strong phenol production, mainly on callus zones. During normal cellular metabolism plants continuously produce ROS but they also simultaneously scavenge these ROS with the help of antioxidant enzymes

and/or metabolites (Watanabe *et al*., 2006 in Younis *et al*., 2010). When explants are cut they exude phenolic compounds that readily oxidize. Oxidized phenolic compounds inhibit enzyme activity and darken the culture medium. Several antioxidants such as ascorbate, cysteine, dithiothreitol, glutathione and tocopherol were successfully used in plants to overcome tissue browning during *Agrobacterium*-mediated transformation (AMT). Dan (2008) recognised two groups of antioxidants used in plant transformation based on their function. The first group consisting of ascorbic acid (AA), cysteine (CYS), dithiothreitol (DTT), lipoic acid, and polyvinylpyrrolidone (PVP), functions to reduce explant necrosis, increase viability of explants, and improve transformation efficiency while the other one, which include glutathione (G), selenite and α-tocopherol, reduces hyperhydricity and ROS and increases transformation efficiency.

Successful AMT methodology for different varieties of *Vicia faba* L. was developed by Jelenić *et al*., 2000; Böttinger *et al*., 2001 and Hanafy *et al*., 2005, but with quite low transformation efficiency (Atif *et al*., 2013). Although, tested cultivars of broad bean were very susceptible to AMT, the regeneration of transgenic broad bean plants still remains the main problem of the practical use of *Agrobacterium*-mediated gene delivery in the broad bean (Jelenić *et al*., 2000). A range of chemical substances with a different nature and action were used for cocultivation during AMT with the aim of enhancing the final efficiency of the transformation protocol (Švábová and Griga, 2008). Generally, these substances work through induction of virulence – acetosyringone (AS), reduction of resistance of the recipient cell (thiol compounds, antioxidants), or a facilitation to penetrate through the cell wall (mechanically or chemically) (Dandekar and Fisk, 2005; Opabode, 2006; Vrbová *et al*., 2013).

The aim of our study was to elaborate efficient and highly reproducible regeneration protocol for faba bean as a basis for application of AMT methodology. Several antioxidants were examined to solve the problems connected with occurrence of phenolic compounds and to prevent browning during the whole regeneration period and transient expression studies after AMT. For the first time in *Vicia faba* L., antioxidants were used in combination with co-cultivation substances (AS, CYS) to test their capability of joint application in genetic transformation.

MATERIAL AND METHODS

Plant material, explants, in vitro systems, culture media

Three low-tannine cultivars of *Vicia faba* L., white-flowering types Merkur, Merlin and colour-flowering type Mistral, were used for the experiments. Seeds of the faba bean were surface sterilized in 75% ethanol for 1 min, in 10% Chloramine T® (Bochemie, Bohumín, CZ) for 20 min and then they were rinsed thrice with sterile deionized water. Sterile germination proceeded on moistened cellulose wadding covered by filter paper in Erlenmayer's flasks (250 ml) in dark for 5–7 days at 22 ± 1 °C. Explants were obtained after excision of ethiolized embryogenic axis from cotyledons and roots, (1) shoot apices (3–5 mm in size) and (2) cotyledonary nodes (5 mm), both containing meristems that were used for the establishment of multiple-shoot culture. Explants (3 × 20 explant per treatments or 2 × 20 explants per treatment) were cultured on the MS macro- and micro- elements with B5 vitamins medium (MSB) (Griga *et al*., 1986), supplemented with 4.5 mg.l^{-1} BAP (6-benzylaminopurine) and 0.019 mg.l^{-1} NAA (1-naphthaleneacetic acid), pH = 5.8 under 16h light photoperiod (56 µmol m^{-2}s^{-1}) at 22 ± 1 °C for 1 month to obtain multiple shoot cultures.

Antioxidants and co-cultivation agents

Three antioxidants were tested in multiple shoot cultures: ascorbic acid (AA; Duchefa Biochemie BV, Haarlem, The Netherlands) (50 mg.l^{-1}), citric acid (CA; Lachner, Neratovice, CZ) (50 mg.l^{-1}), and glutathione (GSH; Duchefa Biochemie BV, Haarlem, The Netherlands) (100 mg.l^{-1}). Antioxidants were added to cultivation agar MSB media (MS macro- and micro- elements, B5 vitamins) (Griga *et al*., 1986), supplemented with 4.5 mg.l^{-1} BAP (6-benzylaminopurine) and 0.019 mg.l^{-1} NAA (1-naphthaleneacetic acid), pH = 5.8 by microfiltration (0.22µm filter unit MILLEX®-MP, Millipore Ireland, Cork, IRL). These antioxidants were also tested in combination with co-cultivation agents acetosyringone (AS; Sigma-Aldrich Chemie, Steinheim, Germany) (20 mg.l^{-1}) and L-cysteine (CYS; Duchefa Biochemie BV, Haarlem, The Netherlands) (25 mg.l^{-1}). In another experimental series, the effect of activated charcoal (ACH; LachNer, Neratovice, CZ) (5 g.l^{-1}) was also tested in multiple shoot cultures on the MSB media with 4.5 mg.l^{-1} BAP (6-benzylaminopurine) and 0.019 mg.l^{-1} NAA (1-naphthaleneacetic acid), pH = 5.8 for the same explants. Observation of explants and evaluation of growth parameters was recorded after one month of culture.

Evaluation of growth parameters

After 4-5 weeks cultivation morphological parameters connected with viability and regeneration capability were assessed – explants weight, shoot weight, shoot number, shoot weight % (weight of shoot / weight of explant × 100). In experiments with the application of activated charcoal, the parameters of rhizogenesis were also evaluated, namely: number of explants with roots, root number per explant, root length and colour, root branching (data not shown), and root weight % (weight of root / weight of explant × 100).

Statistical analysis of quantitative characters

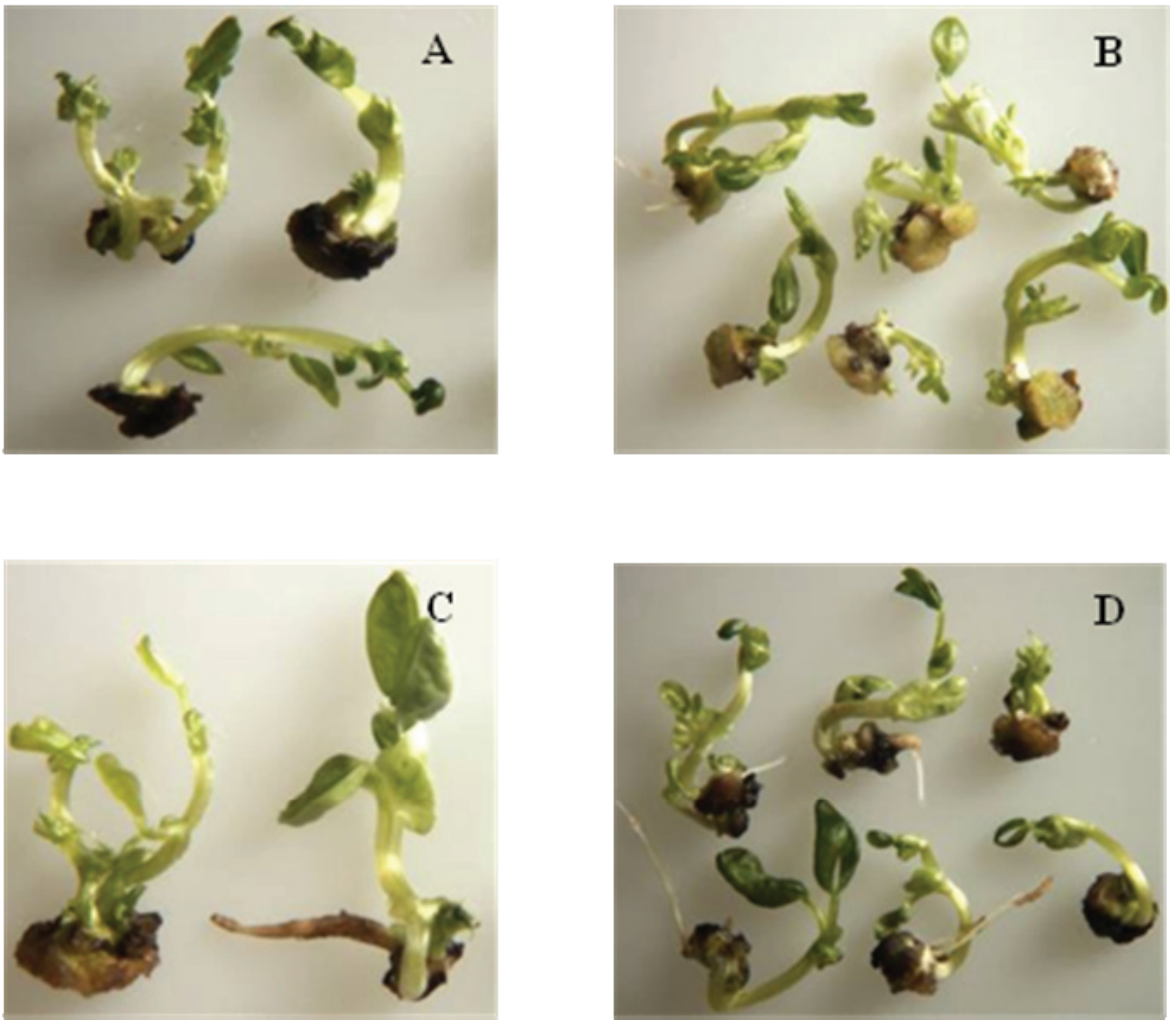
Experiments were designed as 3 × 20 or 2 × 20 explants per treatment. Data were analysed by STATISTICA 8 CZ software (StatSoft, Tulsa, USA), using ANOVA and post-hoc Tukey's HSD test for means at $\alpha = 0.05$.

RESULTS

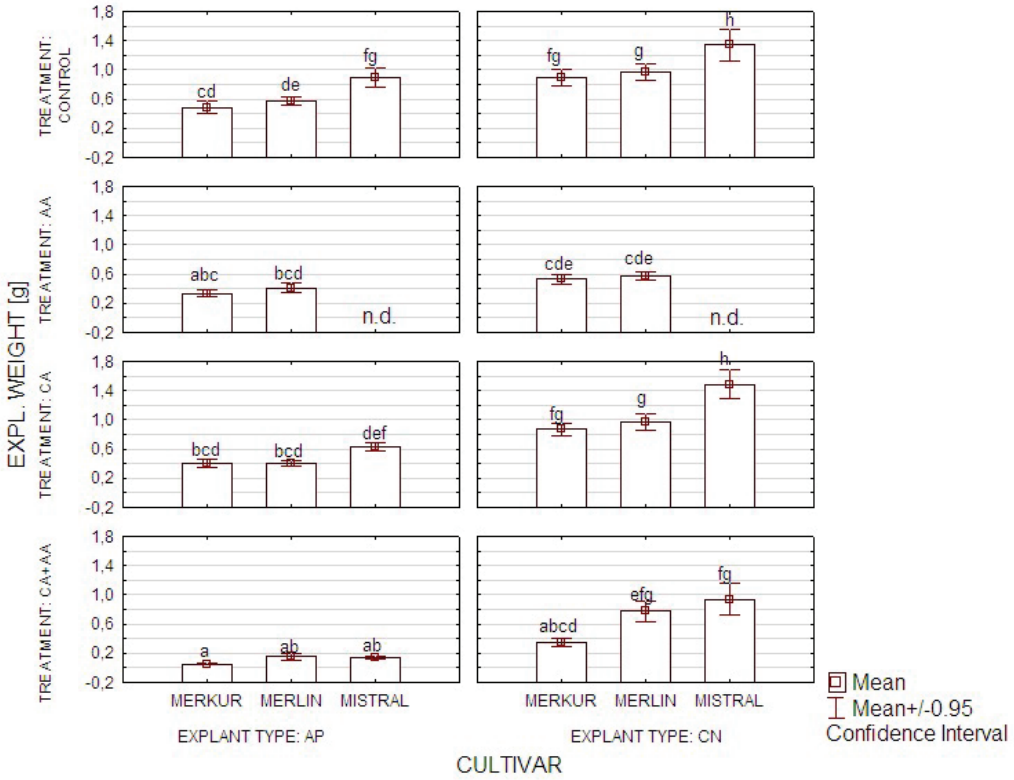
For *in vitro* cultures, the most desirable character is the high number of proliferating shoots competent to rooting and regeneration to the complete fertile plants. In the preliminary experimental series, the effect of AA, CA and GSH on cotyledonary nodes was examined. Callogenesis occurred at all shoot bases by all treatments, but on the media containing CA the calli were smaller and obviously less brown than the calli in experimental variants (Fig. 1). Increase of hyperhydricity was observed as a negative effect, mainly after GSH treatment, when compared to control.

The effect of AA, CA and combination of both antioxidants (CA+AA) was studied in apex and cotyledonary node culture in three faba bean cultivars – Merkur, Merlin and Mistral. After 4 weeks period the explants as a whole, and separately - calli and shoots were weighted. The application of AA affect the weight of explants derived from apices negatively, but not significantly, in cotyledonary node culture the explant weight was significantly decreased. Citric acid treatment did not affect the explant weight which values were similar to the controls. The total explant weight was negatively influenced by combination of both antioxidants – CA+AA, mainly in apex culture where the explants were completely inhibited in growth. In the response of cotyledonary nodes to CA+AA treatment, genotypic variability was recorded (Fig. 2).

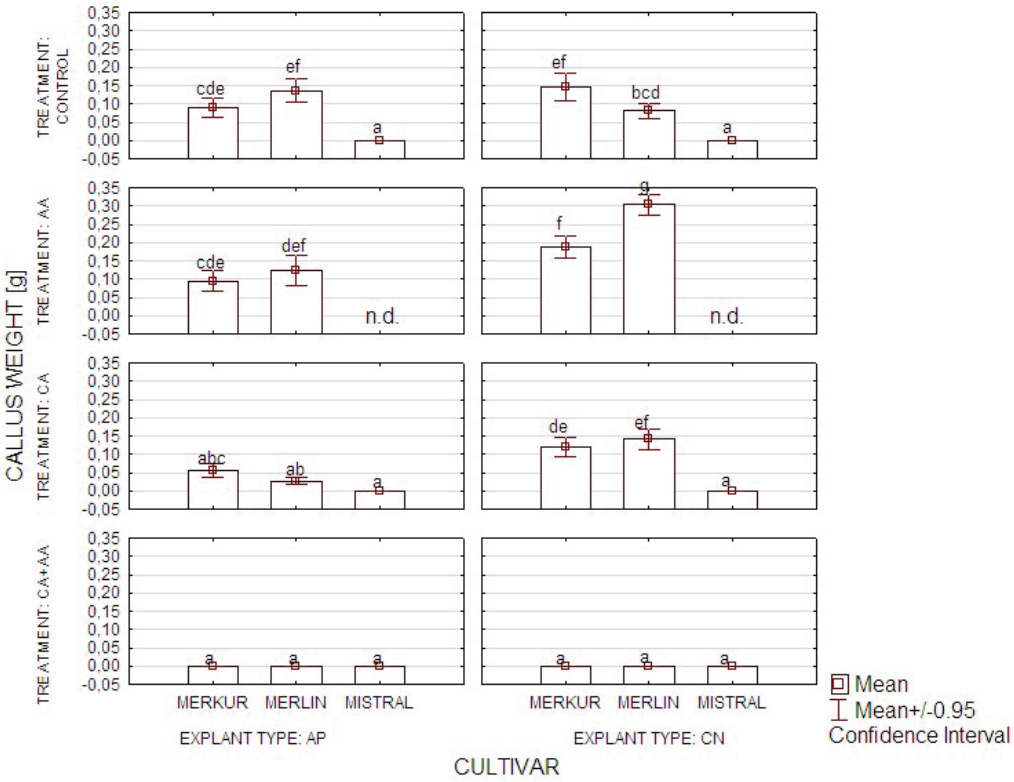
The growth of calli was not recorded by cultivar Mistral, while cultivars Merkur and Merlin formed calli at the basis of explants. It is generally known in leguminous species, that growth of calli inhibits rooting and also the shoot development. The application of AA did not affect calli growth in apex cultures, but in cotyledonary nodes it



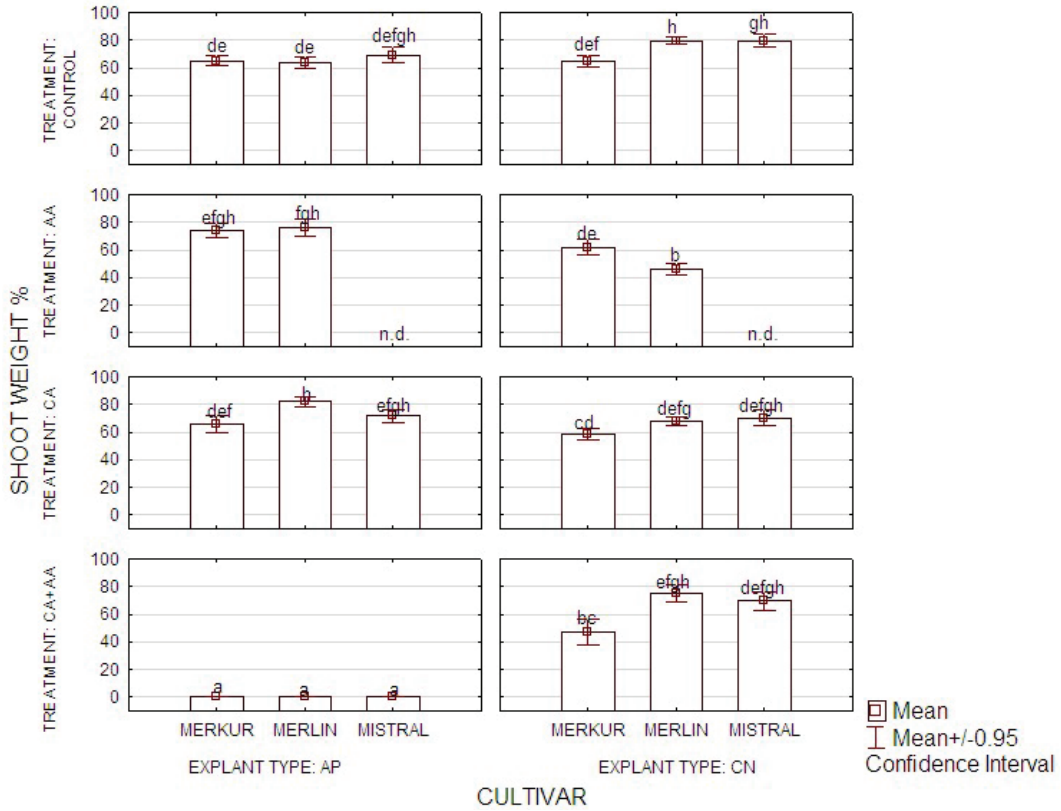
1: *Plant regeneration of faba bean. Effect of antioxidants in one month old node culture of variety Merkur: A – control (without antioxidants), B – citric acid, C – ascorbic acid, D – glutathione*



2: *Explant weight after treatment with AA, CA, and combination of both antioxidants – CA+AA in the three faba bean cultivars and two explant types (AP, CN). Evaluated after four weeks of culture. Different letters mean significant differences assessed by Tukey's HSD.*



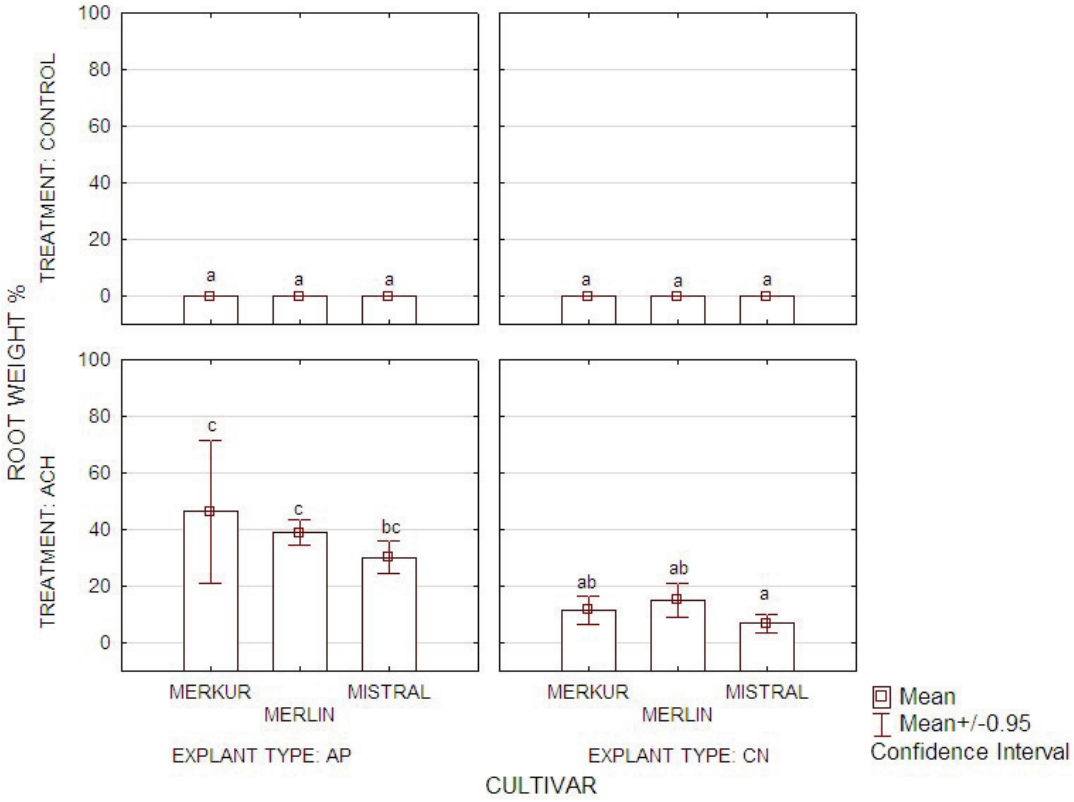
3: *Callus weight after treatment with AA, CA, and combination of both antioxidants – CA+AA in the three faba bean cultivars and two explant types (AP, CN). Evaluated after four weeks of culture. Letters mean groups of significant differences assessed by Tukey's HSD.*



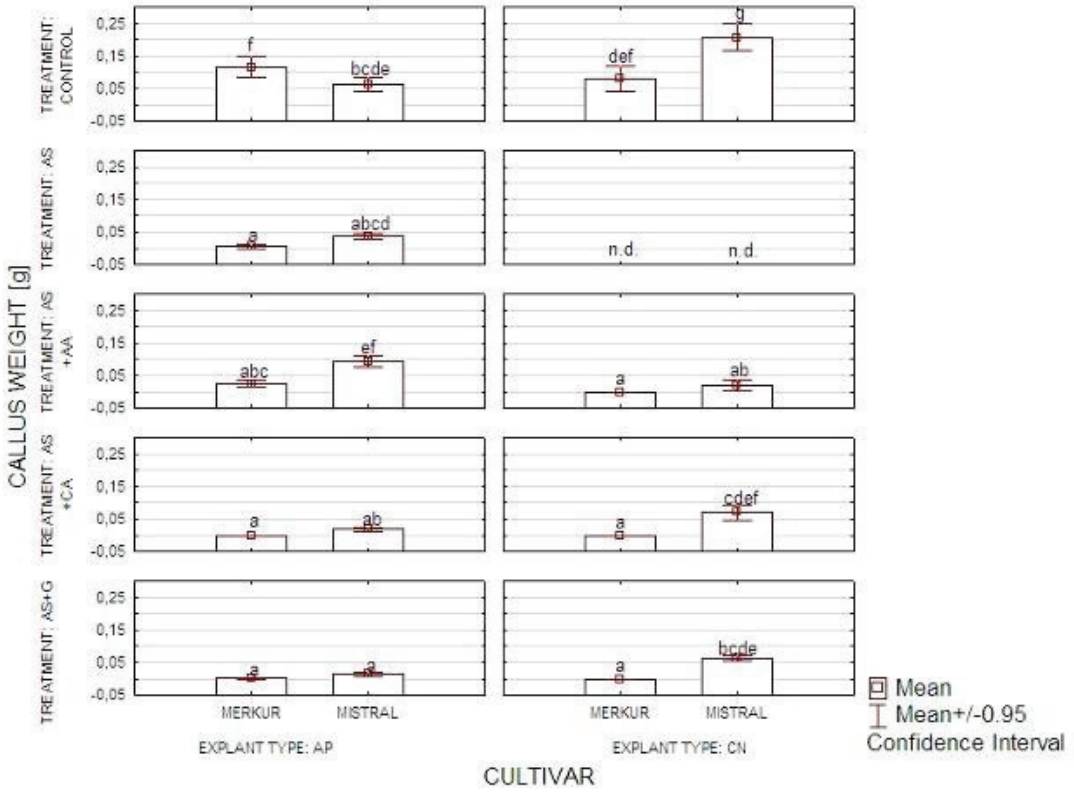
4: *Shoot weight % after treatment with AA, CA, and combination of both antioxidants – CA+AA in the three faba bean cultivars and two explant types (AP, CN). Evaluated after four weeks of culture. Different letters mean significant differences assessed by Tukey's HSD.*

5: *Development of completed plantlets of Vicia faba L. on media with ACH (A – apex, B – cotyledonary nodes)*

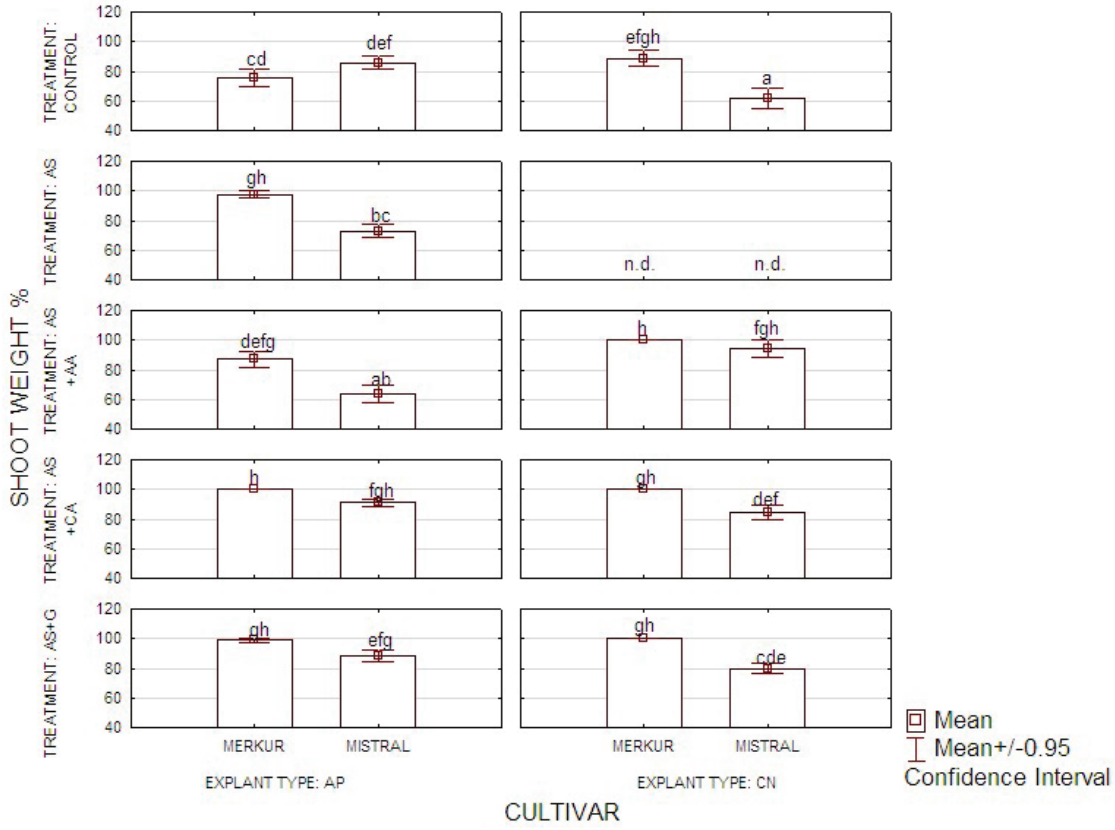
increases statistically significantly the calli weight. Citric acid inhibits the callus formation in apex cultures, and in cotyledonary nodes the response was different possibly due to genotypic variability



6: *Root weight % after treatment with ACH in the faba bean cultivars and two explant types (AP, CN). Evaluated after four weeks of culture. Different letters mean significant differences assessed by Tukey's HSD.*



7: *Callus weight after treatment with AS and combination of AS with antioxidants (AA, CA, G) in the two faba bean cultivars and two explant types (AP, CN). Evaluated after four weeks of culture. Different letters mean significant differences assessed by Tukey's HSD.*



8: *Shoot weight % after treatment with individual AS and combination of AS with antioxidants (AA, CA, G) in the two faba bean cultivars and two explant types (AP, CN). Evaluated after four weeks of culture. Letters mean groups of significant differences assessed by Tukey's HSD.*

(Fig. 3). Moreover, the calli after CA treatment were completely different in the colour and the character as compared to controls. The control calli were brown-black and compact (Fig. 1A), whereas the calli after CA treatment were light green and soft (Fig. 1B). Combined treatment with CA+AA completely eliminates callus growth in all experimental variants.

Shoot weight % was calculated as weight of shoot / weight of explant × 100. This character is decisive for further shoot and whole plant development. The apex culture after treatment with AA and CA resulted in different response in cultivars Merkur and Merlin. Shoot weight % in Merkur was not influenced, while it significantly increased in cultivar Merlin treated with CA. Similarly the reaction in cotyledonary nodes was also genotypicaly different, cultivar Merkur decreased shoot weight % after AA and CA treatment, besides after combined application of CA+AA the decrease of this trait was statistically significant. The situation in Merlin was distinct, AA and CA treatments decreased shoot %. On the opposite, the combination of CA+AA did not cause any effect. The data for cultivar Mistral in both cultures illustrate that application of all variants of antioxidants slightly decreases shoot % (Fig. 4). Genotypic variation in faba bean in response to antioxidants may be influenced also by cultivar flower type – cultivars Merkur and Merlin are white-flowering, and Mistral is colour-flowering type.

Additionally, experiments with the application of activated charcoal were carried out. ACH (5 $g.l^{-1}$) induced root proliferation on media MSB containing 4.5 $mg.l^{-1}$ BAP and 0.019 $mg.l^{-1}$ NAA that are generally used for shoot induction (Fig. 5). Root weight % was calculated as weight of root / weight of explant × 100. Especially in apex cultures the proportion of roots was significantly higher than by controls (Fig. 6), and reached about 40%.

Following experiments proceeded with cultivars Merkur (WF type) and Mistral (CF type). When combination of antioxidants and acetosyringone were applied, decreased callogenesis as compared to control variants was observed at all experimental variants, i.e. AS alone, AS+AA, AS+CA, AS+G (Fig. 7). In the assessment of shoot weight % in apex culture cultivar Merkur reached significantly higher values in all variants than controls. For Mistral negative effect was recorded in variants AS and AS+AA (combination with ascorbic acid), but variants with AS+CA and AS+G did not differ significantly from controls. In cotyledonary node cultures Merkur stayed in all variants on the level of controls, but in Mistral the positive effect after all treatments was recognised (Fig. 8).

In addition to the above application of L-cysteine in combination with AS was examined with no significant effect in all variants of treatments and cultivars (data not shown).

In our subsequent examination, CA also acts very well as antioxidant during GUS staining of reporter *uidA* gene identification. When CA is not used, the staining solution becomes brown-black, making evaluation of *uidA* gene presence impossible. The genotypic variability which made some level of inconsistence of results in our experiments was caused not only with different colour type of cultivars (white- vs. colour-flowered) connected with expected low versus high polyphenol content in tissues, but also with different so-called "plasticity" or "adaptability" for *in vitro* cultures which is better by cultivar Merkur than by Merlin.

DISCUSSION

Phenolic compounds naturally occurring in plants of faba bean (*Vicia faba* L.) play an important role in culture *in vitro* and regeneration of explants. Phenols rapidly accumulating in the cutting side of explants provoke browning, and eventually kill the explants. This study was focused on the application of different antioxidants to find the most suitable type to repress the negative production of phenols and to obtain successful *in vitro* regeneration, outgrowth of shoots, inhibition of callus masses and normal rooting. Abdelwahd *et al.* (2008) tested the addition of several antioxidants and adsorbents (ascorbic acid, active charcoal, polyvinylpyrrolidone) and showed an effective PVP treatment (over-night soaked seeds), for greatly reducing lethal browning in faba bean explants cultured on medium containing TDZ and BAP. They observed positive effects of activated charcoal on the vigour of regenerating shoots and their further ability to root, which is in agreement with our results. They also found a positive effect of ascorbic acid, which was in our experiments assessed as the "second best", but the appearance of explants (smaller light green, and soft calli on the base (Fig. 1) after citric acid treatment made us prefer CA to AA. Citrate in citric acid work as chelating agent and can be used as a prevention agent for browning. We also found that citric acid, applied to the soaking medium, was effective for the prevention of browning during initial excision of explants before inoculation with *Agrobacterium*. On the opposite, glutathion was observed to be negative in faba bean cultures mainly due to its ability to provoke hyperhydricity that always lead to gradual dying of explants.

In *Leguminosae* genetic transformation experiments, co-cultivation agents are succesfully used, but there is no evidence for faba bean. Co-cultivation agents used for the enhancement of AMT efficacy, such as L-cysteine, dithiothreitol or sodium thiosulphate, increase T-DNA delivery, and represent another type of polyphenols influencing the occurrence of browning during the process of regeneration after AMT. In AMT methodology, this important anti-necrotic step of application antioxidants in pre- treatment is generally recommended for reducing oxidative burst (Alimohammadi and Bagherieh-Najjar, 2009).

Against this background, it was deemed necessary to examine the joint application of antioxidants and the co-cultivation treatment. Our experiments showed no effect in Mistral apex- and Merkur cotyledonary-cultures, and increased shoot % Merkur apex-cultures and Mistral cotyledonary-cultures. For ascorbic acid, the effect was either negative or insignificant, except with Mistral cotyledonary culture, which reinforced our preference for citric acid. In our subsequent examination, CA also acted very well as antioxidant during GUS staining of reporter *uidA* gene identification (data not shown). When CA was not used, the staining solution became brown-black; making evaluation of *uidA* gene presence impossible, blue colour was completely overlaid by brown-black impurities in GUS staining solution.

Based on their functional classification, antioxidants are grouped into substances that reduce explant necrosis, increase their viability and improve transformation efficiency (AA, CYS, DTT, lipoic acid, PVP) and those substances that reduce hyperhydricity and ROS and increase transformation efficiency (GLU, selenite, α-tocopherol) (Dan, 2008). In our experiments on *Vicia faba*, glutathion was observed as ineffective to prevent hyperhydricity, suggesting that the response to *in vitro* conditions of faba bean is extremely genotype-dependent and that faba bean in general is excessively sensitive to stress conditions after treatment with chemical substances. Use of antioxidants in faba bean tissue cultures is frequent (Abdelwahd *et al.,* 2008; Ismail *et al.,* 2011, Younis *et al.,* 2010), but their apparent inconsistency is probably not due to "human errors" but rather to these factors above. The differences between plant species (even in the family *Leguminosae*) result in inconvenience to generalize such results and make recommendations for e.g. faba bean or *Leguminosae*. We can nevertheless conclude that there is a need to make preliminary experiments with concrete faba bean genotypes before starting more extensive genetic transformation experiments.

CONCLUSIONS

This paper deals with optimisation of *in vitro* regeneration systems of two explants types for low-tannine cultivars of faba bean – Merkur, Merlin and Mistral based on culturing of shoot apices and cotyledonary nodes with various antioxidants - ascorbic acid, citric acid, glutathione and activated charcoal. This study presents the combined effects of antioxidants with transformation co-cultivation compounds acetosyringone and L-cysteine. In spite of occurrence of genotypically specific responses in faba bean cultivars we can summarize that citric

acid works as the best antioxidant agent and can be applied as additive to media (50 mg.l^{-1} CA), as well as rinsing solution (50 mg.l^{-1} CA) either before or during culture procedure. Individually applied CA did not limit shoot development, but in combination with AA the effect is inhibitory, particularly in apex cultures. Activated charcoal probably blocks the effect of BAP, and result in quite rapid rooting of explants. Citric acid may be used in combination with acetosyringone without undesirable effect on regeneration capability of shoots and development of plants.

The results indicate that *in vitro* cultivation of apex excised from germinated seeds on agar MS media with B5 vitamins, supplemented with 4.5 mg.l^{-1} BAP (6-benzylaminopurine) and 0.019 mg.l^{-1} NAA (1-naphthaleneacetic acid) and antioxidants - citric acid and activated charcoal, co-cultivation agents, L-cysteine and L- acetosyringone are finally decided to use in AMT methodology.

SUMMARY

The experimental study was aimed to achieve accurate regeneration protocol for AMT methodology in faba bean (*Vicia faba* L.). In a case of faba bean only apex of embryonal axis seems to be the one *in vitro* regeneration system capable this requirement to attain and only in the case, that chemical agents helping to sufficient *Agrobacterium* infection and next efficient regeneration of transformed cells would be use. We have been to solved two critical points in AMT methodology, excision of explants and *Agrobacterium* attack on the cuttings that resulted in tissue necrosis. Tested antioxidants and co-cultivation agents would be selected based on current knowledge about transformation protocols in legumes. Our experimental findings recognized to use citric acid, L-cysteine and L-acetosyringone as the helpful agents, but in short term exposition. Then culturing of transfected explants on MSB medium with BAP and NAA would be induced multiplication of axillary shoots at BAP (4.5 mg.l^{-1}) or would be necessary to use other higher concentration of BAP or more efficient growth regulator. In the *in vitro* regeneration process we need recognized to use of activated charcoal as the best inducer of rooting outgrowing shoots of faba bean.

Acknowledgement

This study was supported by grants: MSMT No. 1M06030, NAZV No. 91A229. The authors would like to thank Prof. Sergio J. Ochatt for collaboration and valued recommendations. Excellent technical support of Jana Ščamburová for providing of technician work is greatly acknowledged.

Abbreviations:

AA – ascorbic acid
ACH – activated charcoal
AMT – *Agrobacterium*-mediated transformation
AP – culture initiated from shoot apices
AS – acetosyringone
BAP – benzylaminopurine
CA – citric acid
CYS – L-cysteine
FW – fresh weight
GSH – glutathion
HSD – honestly significant difference
MSB – MS medium with B5 vitamins
NAA – naphtalene acetic acid
ND – culture initated from cotyledonary nodes
n.d. – not done
ROS – reactive oxygen species
TDZ – thidiazurone.

REFERENCES

ABDELWAHD, R., HAKAM, N., LABHILILI, M., UDUPA, S., 2008: Use of an adsorbent and antioxidants to reduce the effects of leached phenolics in *in vitro* plantlet regeneration of faba bean. *African J. Biotechnol.*, 7, 8: 997–1002. ISSN 1648-5315.

ALIMOHAMMADI, M., BAGHERIEH-NAJJAR, M. B., 2009: *Agrobacterium*-mediated transformation of plants: Basic principles and influencing factors. *African J. Biotechnol.* 8, 20: 5142–5148. ISSN 1684-5315.

ANWAR, F., ALGHAMDI, S. S., AMMAR, M. H., SIDDIQUE, K. H. M., 2011: An efficient *in vitro* regeneration protocol for faba bean (*Vicia faba* L.). *Journal of Medicinal Plants Research*, 5, 20: 6460–6467. ISSN 1996-0875.

ATIF, R. M., PATAT-OCHATT, E. M., SVABOVA, L., ONDREJ, V., JACAS, L., GRIGA, M., KLENOTICOVA, H., OCHATT, S. J., 2013: Gene transfer in legumes. In: LÜTTGE, U., BEYSCHLAG W., FRANCIS, D., CUSHMAN, J. (Eds.), Progress in Botany 74: Springer-Verlag Berlin Heidelberg, 37–100, ISBN 978-3-642-30967-0-2.

BÖTTINGER, P., STEINMETZ, A., SCHIEDER, O., PICKARDT, T., 2001: *Agrobacterium*-mediated transformation of *Vicia faba*. *Mol. Breeding,* 8, 3: 243–254. ISSN 1380-3743.

DAN, Y., 2008: Biological functions of antioxidants in plant transformation. *In Vitro Cell. Dev. Biol.-Plant,* 44, 3: 149–161. ISSN 1054-5476.

DANDEKAR, A., FISK, H. J., 2005: Plant transformation. *Agrobacterium*-mediated gene transfer. *Methods Mol. Bol.*, 286: 35–46. ISSN 1064-3745.

FAKHRAI, H., FAKHRAI, F., EVANS, P. K., 1988: *In Vitro* Culture and Plant Regeneration *in Vicia faba* subsp. Equina (var. Spring Blaze). *J. Exp. Bot.,* 40, 7: 813–817. doi:10.1093/jxb/40.7.813.

GRIGA, M., 1988: The study of differences in regeneration potential of various *Vicia faba* L. genotypes in meristem-tip culture *in vitro*. *Plant Production*, 34, 6: 613–626. ISSN 0370-663X.

GRIGA, M., KLENOTIČOVÁ, H., 1994: Plant regeneration in callus cultures of faba bean (*Vicia faba* L.). *Plant Production*, 40, LXVII: 697–709. ISSN 0370-663X.

GRIGA, M., KUBALÁKOVÁ, M., TEJKLOVÁ, E., 1987: Somatic embryogenesis in *Vicia faba* L. *Plant Cell, Tissue and Organ Culture,* 9, 2: 167–171. ISSN 0167-6857.

GRIGA, M., TEJKLOVÁ, E., NOVÁK, F. J., KUBALÁKOVÁ, M., 1986: *In vitro* clonal propagation of *Pisum sativum* L. *Plant Cell, Tissue and Organ Culture,* 6, 1: 95–104. ISSN 0167-6857.

HANAFY, M., PICKARDT, T., KIESECKER, H., JACOBSEN, H.-J., 2005: *Agrobacterium*-mediated transformation of faba bean (*Vicia faba* L.) using embryo axes. *Euphytica,* 142, 3: 227–236. ISSN 0014-2336.

JELENIĆ, S., MITRIKESKI, P. T., PAPEŠ, D., JELASKA, S., 2000: *Agrobacterium*-mediated transformation of broad bean *Vicia faba* L. *Food Technol. Biotechnol.*, 38, 3: 167–172. ISSN 1330-9862.

MOHAMED, M. F., READ P. E., COYNE, D. P., 1992: Dark preconditioning, CPPU, and TDZ promote shoot organogenesis on seedling node explants of common and faba beans. *J. Amer. Soc. Hort. Sci.*, 117, 4: 668–672. ISSN 0003-1062.

OPABODE, J. T., 2006: *Agrobacterium*-mediated transformation of plants: emerging factors that influence efficiency. *Biotechnology and Molecular Biology Review*, 1, 1: 12–20. ISSN 1538-2273.

PICKARDT, T., 2003: *In vitro* regeneration and genetic transformation of *Vicia* species. In: *Applied Genetics of Leguminosae Biotechnology*. Dordrecht: Kluwer Academic Publishers, 121–131. ISBN 1-4020-1485-6.

SELVA, E., STOUFFS, B., BRIQUET, M., 1989: *In vitro* propagation of *Vicia faba* L. by micro-cutting and multiple shoot induction. *Plant Cell, Tissue and Organ Culture,* 18, 2: 167–179. ISSN 0167-6857.

ŠVÁBOVÁ, L., GRIGA, M., 2008: The effect of cocultivation treatments on transformation efficiency in pea (*Pisum sativum* L.). *Plant Cell, Tissue and Organ Culture,* 95, 3: 293–304. ISSN 0167-6857.

TEJKLOVÁ, E., GRIGA, M., NOVÁK, F. J., 1984: Hormonal regulation of growth of field bean (*Vicia faba* L.) shoot apices in *in vitro* culture. *Plant Production* 30, LVII: 543–550. ISSN 0370-663X.

VRBOVÁ, M., KOTRBA, P., HORÁČEK, J., SMÝKAL, P., ŠVÁBOVÁ, L., VĚTROVCOVÁ, M., SMÝKALOVÁ, I., GRIGA, M., 2013: Enhanced accumulation of cadmium in *Linum usitatissimum* L. plants due to overproduction of metallothionein α-domain as a fusion to β-glucuronidase protein. *Plant Cell, Tissue and Organ Culture,* 112, 3: 321–330. ISSN 0167-6857.

WATANABE, K., YAMADA, N., TAKEUCHI, Y., 2006: Oxidative DNA damage in cucumber cotyledons irradiated with ultra-violet light. *Journal of Plant Resesearch,* 119, 3: 239–246. ISSN 0918-9440.

WOJTASZEK, P., 1997: Oxidative burst: an early plant response to pathogen infection. *Biochemical Journal,* 322, 3: 681–692. ISSN 0264-6021.

YOUNIS, M. E.-B., HASANEEN, M. N. A.-G., ABDEL-AZIZ, H. M. M., 2010: An enhancing effect of visible light and UV radiation on phenolic compounds and various antioxidants in broad bean seedlings. *Plant Signaling & Behavior*, 5, 10: 1197-1203. http://dx.doi.org/10.4161/psb.5.10.11978.

Address

RNDr. Helena Klenotičová, Ing. Iva Smýkalová, PhD., Ing. Lenka Švábová, PhD., RNDr. Miroslav Griga, CSc., Plant Biotechnology Department, Agritec Plant Research, Zemědělská 16, 787 01 Šumperk, Czech Republic, e-mail: klenoticova@agritec.cz, smykalova@agritec.cz, svabova@agritec.cz, griga@agritec.cz