

Application of sonication-assisted *Agrobacterium*-mediated transformation in *Chenopodium rubrum* L.

J.I. Flores Solís, P. Mlejnek, K. Studená, S. Procházka

Mendel University of Agriculture and Forestry in Brno, Czech Republic

ABSTRACT

Chenopodium rubrum belongs to the plant species in which standard *Agrobacterium*-mediated transformation procedures remain inefficient. We demonstrate that the employment of sonication-assisted *Agrobacterium*-mediated transformation (SAAT) effectively enhanced transient expression of GUS gene coding for β -glucuronidase in *Chenopodium rubrum*. Further the results indicated that the age of seedlings is one of the limiting factors affecting the potency of *Agrobacterium tumefaciens* infection. Histochemical detection of β -glucuronidase activity revealed that two-days-old seedlings were much more susceptible to infection than ten-days-old ones. According to our results SAAT technology could provide an efficient tool for obtaining stable transformants when applied to two-days-old seedlings of *Chenopodium rubrum*.

Keywords: *Agrobacterium tumefaciens*; strain EHA 105; *uidA*; GUS; β -glucuronidase

A transformation system based on the Ti plasmid of *Agrobacterium tumefaciens* has become the major tool for transfer of foreign genes into plants to carry out gene expression studies and to attempt to obtain improved plant varieties of potential agricultural or commercial interest. The first plant transformed by the above-mentioned method was recovered in the middle of 1980s (Horsch et al. 1985). Since then a number of plant species has successfully been transformed by *Agrobacterium*. However, transformation efficiency was lowered by the lack of amenability of some plant species to the *Agrobacterium*. In addition to *Agrobacterium*-mediated transformation, direct DNA transformation based on the invention of a gene gun, i.e. microprojectile bombardment, was developed by Klein et al. (1987). Using this technique it was possible to obtain plant transformants at a high frequency. The main drawback of the direct transformation method is regeneration of mature plants since plant cell protoplasts must be used for transgene introduction.

Another transformation method that omits *in vitro* manipulation was developed by Feldmann and Marks (1987). They cultivated seeds of *Arabidopsis thaliana* with *Agrobacterium tumefaciens* harbouring a co-integrated plasmid to obtain transformed progeny. The use of vacuum-mediated *Agrobacterium* transformation in combination with binary vectors instead of less efficient co-integrated plasmids dramatically simplified transformation procedures since adult plants can be utilised (Bechtold et al. 1993). In this method, *Agrobacteria* are delivered into the intercellular space under vacuum. Although the involved mechanism is not known in detail, it is likely that transformation occurred later during floral development because all transformants were hemizygous and contained different T-DNA inserts (Bechtold et al. 1993).

Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) as an efficient *Agrobacterium*-based transformation technology was recently reported by Trick and Finer (1997). This method consists of subjecting the target plant tissue to brief periods of ultrasound while immersed in an *Agrobacterium* suspension. SAAT overcomes certain barriers such as the host specificity and the inability of *Agrobacterium* to reach proper cells in the target tissues. It also enhances DNA transfer in diverse plant groups including dicots, monocots, and gymnosperms. It is likely that the enhanced transformation rates using SAAT result from micro-wounding both on the surface and deep within the target tissue. Therefore, unlike other transformation methods, this system also has the potential to transform meristematic tissue buried under several cell layers (Trick and Finer 1997).

The species *Chenopodium rubrum* has been widely used for model studies of many basic physiological functions in plants (e.g. Vondráková et al. 1998, Wolf et al. 2001). However, the application of transgenic technology to this species for investigations of gene expression control is not possible as this species resists to standard procedures for plant transformation. According to our knowledge, there are only few reports describing transformation of the genus *Chenopodium*, although mature plants were not recovered. Thus, Komari (1990) described the transformation of cultured cells of *Chenopodium quinoa* using binary vectors. Electroporation of *Chenopodium quinoa* protoplasts with viral RNAs for the study of gene expression was applied by Jung et al. (1992).

In this study we have demonstrated that SAAT method provided satisfactory results also in *Chenopodium rubrum*, however, its efficiency was substantially limited by the age of plant.

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MATERIAL AND METHODS

Plant material. *Chenopodium rubrum* L., selection 374. Seeds were sterilised in a mixture of ethanol, hydrogen peroxide and distilled water (5:5:2) for 1 minute, rinsed once with distilled water, immersed into a solution of 15% NaCl for 20 minutes, rinsed with sterile water again (3×) and placed on a MS medium for cultivation. Complete germination was promoted by changing the temperature as follows: 32°C for 12 h, 10°C for 12 h and 32°C for 12 h under constant illumination (Cumming et al. 1965).

Agrobacterium strain. Hypervirulent *Agrobacterium tumefaciens*, strain EHA 105, in conjunction with the plasmid pGT89, containing the *bar* and *nptIII* genes that confer resistance to phosphinothricin (PPT) and kanamycin, as selection markers, and GUS (*uidA*) gene coding for β -glucuronidase under double-enhancer 35S promoter, as a reporter gene, was used for transformation (generously provided by Samantha Bean, John Innes Centre, Norwich, England).

Agrobacterium cultivation. 5 ml of medium for cultivation (sucrose 10 g/l, casein hydrolysate 8 g/l, yeast extract 4 g/l, KH_2PO_4 2 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g/l, pH = 6.8) supplemented with kanamycin (50 $\mu\text{g/ml}$) was inoculated with a single colony of the *Agrobacterium* and cultivated aerobically overnight. Next day bacteria were transferred into 45 ml of fresh medium supplemented with 50 $\mu\text{g/ml}$ kanamycin and further cultivated until the appropriate density was obtained.

Transformation procedure. 50 ml of exponentially growing *Agrobacteria* of the optical density 1.0 ($\text{OD}_{600} = 1.0$) were pelleted by centrifugation (3500 rpm/15 min at 20°C) and resuspended in 10 ml of medium for co-cultivation (MS salts, B5 medium, pH = 5.8, supplemented with acetosyringone 100 μM). Approximately 60 two-days-old seedlings (or 30 ten-days-old plants) were transferred into a 15ml tube containing 5 ml of resuspended *Agrobacteria*. The tube was immersed into a bath sonicator (model Transsonic T 310, HF-frequency: 35 KHz, ELMA, Germany), the lower part of the tube touching the depth of the bath, and it was subjected to sonication for 75 seconds. The seedlings were removed from the tube, placed on a filter paper to remove the excess of bacteria and placed onto a solid MS medium for two-day co-cultivation under constant illumination, at 27°C. In a single experiment approximately 200 seedlings (in each group) were treated by sonication. The total number of seedlings used was 1217 and 1165 for two-days-old and ten-days-old seedlings, respectively.

Histochemical detection of GUS expression. β -glucuronidase activity was detected using staining with 5-bromo-chloro-3-indolyl β -D-glucuronide, as described elsewhere (Potrykus and Spangenberg 1995). Briefly, plants were incubated under constant illumination in the staining solution (100mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.0, 10mM Na_2EDTA , 5mM $\text{K}_4[\text{Fe}(\text{CN})_6]$, 5mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, 0.1% v/v Triton X-100, and 0.3% w/v 5-bromo-chloro-3-indolyl β -D-glucuronide (Duchefa) overnight, at 27°C. To improve the infiltration of staining solution, vacuum

(200 mBar) was applied repeatedly (at least twice for 3 min). The stained plants were then fixed in a fixation solution (5% v/v formaldehyde, 5% v/v acetic acid, 20% v/v ethanol) for 15 min at room temperature. The fixed tissue was washed with 70% ethanol (v/v) three times and examined for staining under microscope.

RESULTS

Since standard transformation procedures provided unsatisfactory results according to our experience, we adopted SAAT method to enhance the efficiency of *Agrobacterium*-based transformation procedure in *Chenopodium rubrum*. Preliminary results indicated that the application of SAAT method dramatically increased transient expression of GUS gene coding for β -glucuronidase in young seedlings of *Chenopodium rubrum*. Detailed analysis revealed that gene transfer efficiency decreased with the increasing age of seedlings. Two-days-old seedlings appeared to be the most sensitive to agrobacterial infection (not shown). To demonstrate different susceptibility of *Chenopodium rubrum* seedlings to gene transfer mediated by *Agrobacterium tumefaciens* with respect to their age histochemical detection of β -glucuronidase activity in two-days-old and ten-days-old seedlings was carried out. The frequency of occurrence of seedlings transiently expressing GUS gene was approximately 1.6 times higher in two-days-old seedlings than in ten-days-old ones, as shown in Figure 1. We also observed a large difference in the distribution of β -glucuronidase activity between both groups. While in two-days-old seedlings β -glucuronidase activity was detected besides cotyledons and hypocotyls also in the apex (Figures 2b–f and 3a), in ten-days-old seedlings β -glucuronidase activity was detected only in leaves and stems (Figures 2h–k and 3b). None of the 1165 ten-days-old seedlings subjected to SAAT method exhibited β -glucuronidase activity in the apex (not shown). The occurrence of β -glucuronidase activity in apical cells of two-days-old seedlings seems to be of great importance since apical cells give rise to floral organs, thus increasing the probability of gene transfer to progeny. In addition, the tissues transiently expressing GUS gene formed much larger areas after histochemical staining in two-days-old seedlings than in ten-days-old ones (Figure 2) further indicating higher susceptibility of younger seedlings to agrobacterial infection following the application of SAAT method.

In conclusion our results suggest that SAAT method applied to two-days-old seedlings could provide an efficient tool for obtaining transformed progeny in the genus *Chenopodium rubrum*, otherwise resistant to standard transformation procedures.

DISCUSSION

An enormous effort to transform *Chenopodium rubrum* was invested in our laboratory. In the first experiments we

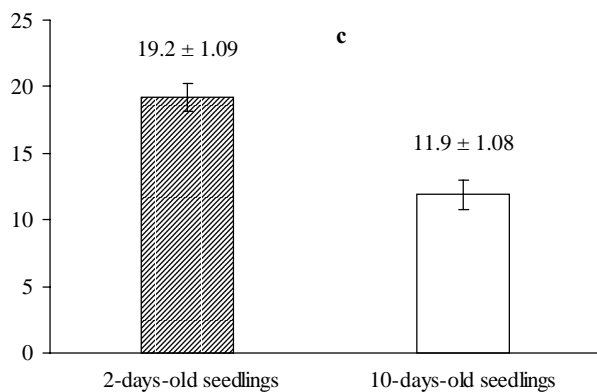
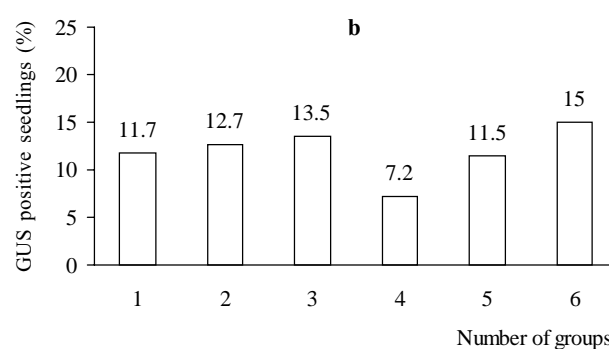
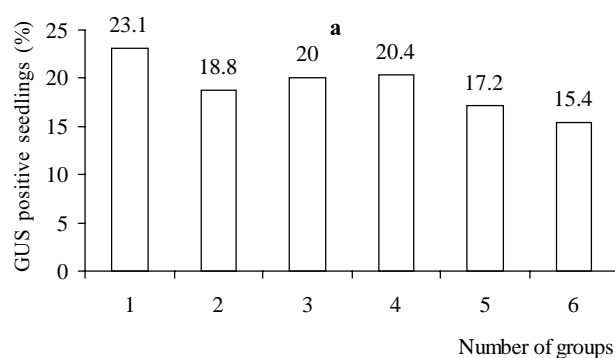


Figure 1. Comparison of the frequency of occurrence of β -glucuronidase activity in two-days and ten-days-old seedlings subjected to SAAT method

a – 1217 of two-days-old seedlings were divided into six groups (approximately 200 into each) and treated separately, the columns represent percentage of positive seedlings in each group

b – 1165 of ten-days-old seedlings were divided into six groups (approximately 200 into each) and treated separately, the columns represent percentage of positively stained seedlings in each group

c – average numbers of positive two-days (solid column) and ten-days-old (open column) seedlings with standard deviations

employed stem, cotyledon, and leave sections that provided satisfactory results as far as the transient expression of GUS gene/gene transfer was concerned. However, we failed to regenerate mature plants (unpublished results).

Our results suggest that *Chenopodium rubrum* is resistant to standard transformation procedures such as the method employing co-cultivation of germinating seeds with *Agrobacterium* (Feldman and Marks 1987). This conclusion was based on the finding of zero or negligible β -glucuronidase activity in *Chenopodium* tissue following the treatment (not shown). Similarly, we obtained negative results using a relatively new approach developed by Bechtold et al. (1993) that is based on vacuum infiltration into an adult plant in a suspension of *Agrobacterium tumefaciens* (not shown). In the experiments mentioned above we employed two different strains of *Agrobacterium tumefaciens*, strain LBA4404 and hypervirulent strain EHA 105; in both cases with negative results (not shown).

To overcome the barriers that prevent gene transfer from *Agrobacterium* to *Chenopodium* cells we used the SAAT method. Indeed, the application of SAAT method enabled to transform many plant species including monocots, dicots, and gymnosperms where other approaches failed (Trick and Finer 1997). The exposure of *Chenopodium rubrum* seedlings to brief periods of ultrasound in the presence of *Agrobacterium tumefaciens* immensely enhanced the T-DNA transfer process judging from the increased transient expression of GUS gene in treated plants (Figures 1–3). This effect is probably due to the energy of ultrasonic frequency that caused micro-wounding both on the tissue surface and deep inside the tissue. Wounded tissue allows the bacterium to infect

target cells. Besides that, wounded tissue could produce phenolic substances thereby mediating the binding accessibility of *Agrobacterium* to the cell surface (Stachel et al. 1985). Our results indicated that gene transfer is much more effective in younger seedlings than in older ones for unknown reasons (Figures 1–3). We can only speculate that decreased susceptibility of older seedlings to gene transfer might be due to their better developed constitution, which prevents plants from the agrobacterial infection. The possibility that older seedlings are more resistant to micro-wounding induced by sonication is unlikely since the prolonged sonication period had no effect on transient expression of GUS gene (not shown). Nevertheless, our results clearly indicate that the age of plant could be a critical factor that substantially affects the efficiency of gene transfer at least in *Chenopodium rubrum* (Figures 1–3). The higher susceptibility of two-days-old seedlings to agrobacterial infection is of both quantitative (Figure 1) and qualitative (Figures 2 and 3) nature. The qualitative aspect of this difference, i.e. occurrence of β -glucuronidase activity in the apex of two-days-old seedlings entitles us to expect the occurrence of GUS gene in progeny with relatively high probability. Indeed, all the organs of mature plant including leaves, stem and gametes originate in the apex. The susceptibility of apex to agrobacterial infection decreases with the age of seedlings. Thus, in ten-days-old seedlings it is very unlikely to expect transfer of GUS gene to progeny since its occurrence was observed neither in apex nor in axillary buds (not shown).

The transient GUS expression is localised on the parts of the seedlings that were not in contact with solid MS medium. A similar spatial pattern in the distribution of

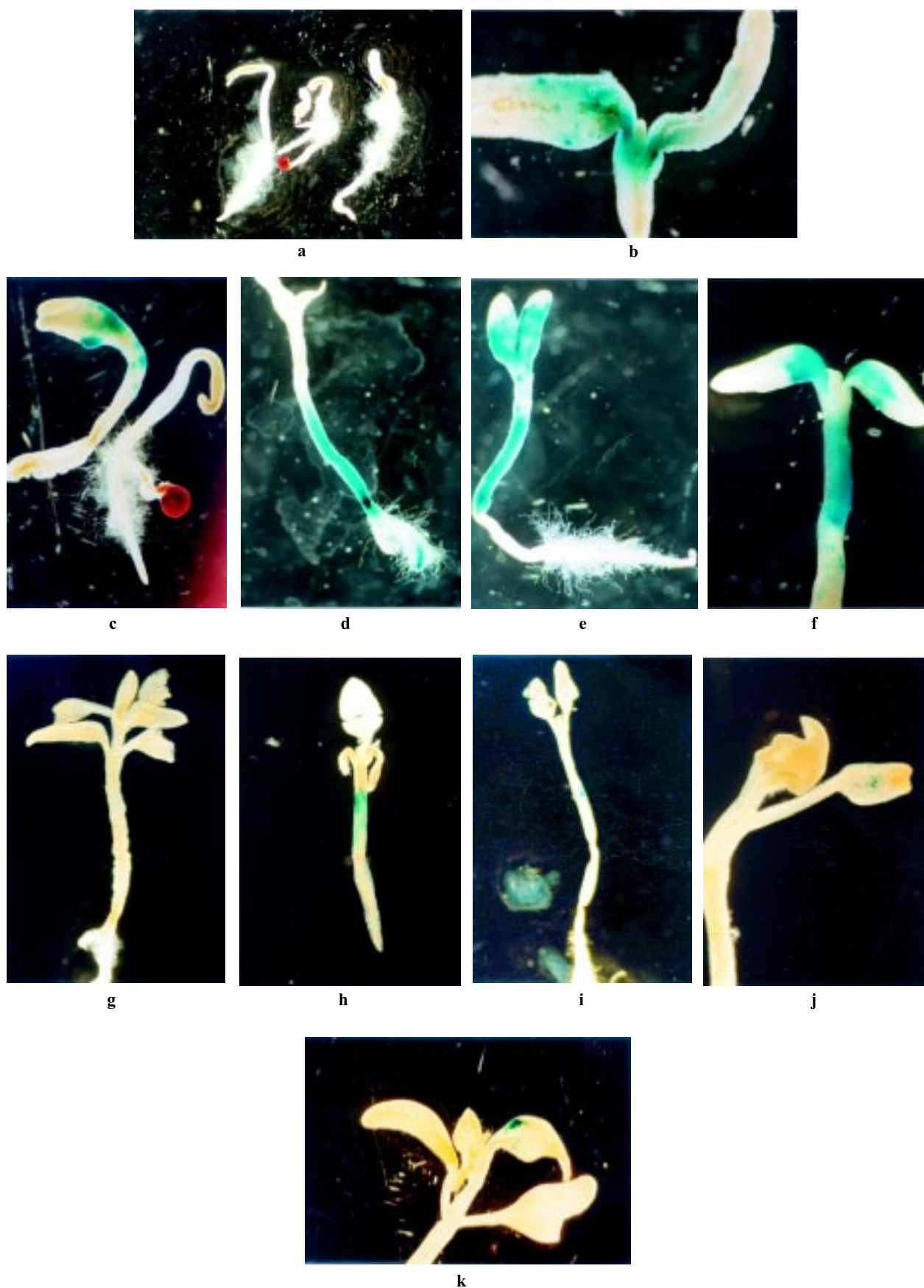


Figure 2. Histochemical staining of β -glucuronidase activity in plant tissues of two-days and ten-days-old plants subjected to SAAT method; two-days-old seedlings were subjected to SAAT method, co-cultivated on solid MS medium for two days and stained as described in Material and Methods (**b** – cotyledons and apex, **c** – cotyledons, **d** – hypocotyl, **e**, **f** – cotyledons and hypocotyl); ten-days-old seedlings were subjected to the same procedure (**h**, **i** – stems, **j**, **k** – leaves); seedlings treated with *Agrobacterium tumefaciens* lacking plasmid pGT89 were used as control (**a**, **g**); the figures represent typical examples

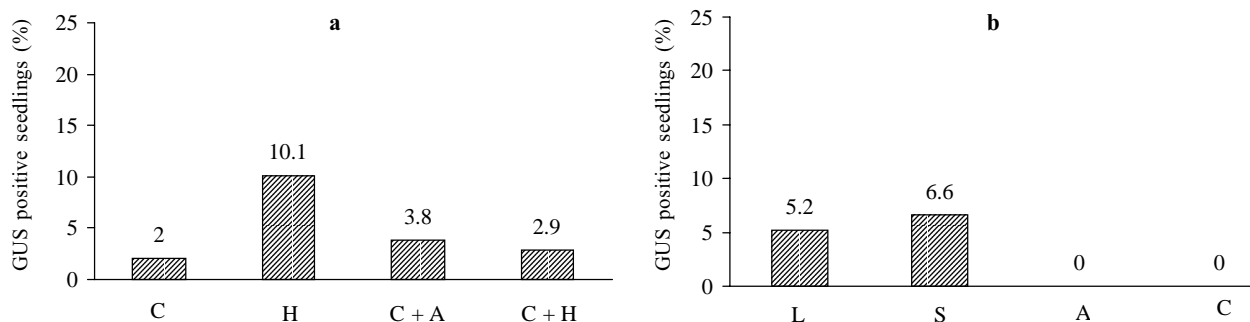


Figure 3. Relative distribution of β -glucuronidase activity in plant tissues of two-days and ten-days-old plants subjected to SAAT method; the columns represent the percentage of seedlings with positive β -glucuronidase activity in the tissues of seedling organs as indicated

a – cotyledons (C), hypocotyls (H), cotyledons with apex (C + A) and cotyledons with hypocotyls (C + H) in two-days-old seedlings
b – leaves (L), stems (S), cotyledons (C), and apex (A) in ten-days-old seedlings

transient GUS gene expression was reported by Santarém et al. (1998) in immature soybean cotyledons subjected to SAAT method. This finding probably reflects an inhibitory effect of MS media, the pH value of which was adjusted to 5.8 (our case) and to 7.0 (Santarém et al. 1998). Indeed, it was reported that the pH value higher than 5.2 exerts an inhibitory effect on *vir* gene induction (Stachel et al. 1986). Therefore, the pH change of growth media towards acidic values could increase susceptibility of plants to agrobacterial infection.

In the present study we demonstrated that SAAT method could provide an efficient tool also for transformation of genus *Chenopodium*. However, our results indicated that the efficiency of this method strongly depends on the age of seedlings. The highest susceptibility to agrobacterial infection was observed in two-days-old seedlings and then it gradually decreased with the age of seedlings.

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ABSTRAKT

Využití ultrazvuku při agrobakteriální transformaci merlíku červeného *Chenopodium rubrum* L.

Merlík červený (*Chenopodium rubrum*) patří mezi rostlinné druhy, u kterých selhávají standardní transformační postupy zprostředkované agrobakteriemi. Ověřili jsme, že použití metody SAAT (sonikací usnadněná transformace zprostředkovaná agrobakteriemi) velmi účinně zvyšuje expresi GUS genu kódujícího β -glukuronidázu (reportérový gen) u merlíku červeného. Výsledky ukazují, že stáří rostlin je jedním z limitujících faktorů, jež ovlivňují účinnost infekce agrobakteriemi. Z výsledků histochemické detekce enzymové aktivity β -glukuronidázy vyplývá, že dvoudenní rostliny jsou mnohem více citlivé k infekci než rostliny desetidenní. Naše výsledky nasvědčují tomu, že použití metody SAAT u dvoudenních rostlin merlíku červeného by mohlo být vhodným nástrojem k získávání stabilních transformantů u tohoto rostlinného druhu.

Klíčová slova: *Agrobacterium tumefaciens*; řetězec EHA 105; *uidA*; GUS; β -glukuronidáza

Corresponding author:

Mgr. Jorge I. Flores Solís, Mendelova zemědělská a lesnická univerzita v Brně, Zemědělská 1, 613 00 Brno, Česká republika
tel.: + 420 545 133 011, fax: + 420 545 133 025, e-mail: flores@mendelu.cz
