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THE EFFECTS OF CEFOTAXIME AND SILVER THIOSULPHATE ON *IN VITRO* CULTURE OF *SOLANUM CHACOENSE*

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E. RÁKOSY-TICAN[1], C. M. AURORI[1], A. AURORI[1]
[1]babeş-Bolyai University, Faculty Of Biology and Geology
Plant Genetic Engineering Group . Clinicilor str. 5-7, RO-400006 Cluj-Napoca, Romania
Correspondence to: Elena Rákosy-Tican, BABES-BOLYAI UNIVERSITY, FACULTY OF BIOLOGY AND GEOLOGY , Clinicilor str. 5-7, RO-400006 Cluj-Napoca, Romania, Tel. (0040) 264 431878, Fax. (0040) 264 591906, E-mail: lrakosy@hasdeu.ubbcluj.ro / arina5744@yahoo.com
Proofs to: Elena Rákosy-Tican, Clinicilor str. 5-7 RO-400006 Cluj-Napoca Romania

Abstract

Solanum chacoense Bitt., a highly polymorphic, tuber-bearing, wild diploid species holds great promise for the genetic improvement of cultivated potato as a reservoir of resistance genes to insects (Colorado potato beetle), fungi (common scab), bacterial wilt and soft rot, potato virus X and potato virus Y, root-knot nematodes and abiotic stress such as heat and drought. This is why any improvement of in vitro culture of this species is desirable.The goal of our study was to assess the effects of cefotaxime and silver thiosulphate (STS) on in vitro growth of Solanum chacoense shoots regenerated directly from the axillary buds of nodal explants It is shown that cefotaxime stimulates in vitro growth of S. chacoense. Regardless of the concentration, mainly root growth and leaf fresh weight were significantly stimulated. Moreover, it is shown that maintaining the antibiotic stock solution in the freezer for a longer period of time (more than 3 weeks) has reduced the positive effect on plant growth. Silver thiosulphate added to the culture media stimulated mainly leaf fresh weight that might have a beneficial effect on mesophyll protoplast isolation. Since during repeated subcultures a bacterial-induced dwarf somaclone was selected, cefotaxime was also used to eliminate internal bacterial contamination and to reverting the dwarf somaclone to normal growth.

Keywords: antibiotics, bacterial induced dwarfism, ethylene inhibitors, *in vitro* growth

Introduction

Wild *Solanum* tuber-bearing species related to the cultivated potato represent an important reservoir of genetic diversity (Hawkes, 1990 [1]). *Solanum chacoense* Bitt. is a highly polymorphic, self-incompatible tuber-bearing diploid species (2n = 2x = 24). It holds great promise for the genetic improvement of cultivated potato (Hawkes & Hjerting, 1989 [2]). By spreading as a field weed in lowland pastures through South Bolivia, North and Central Argentina, Paraguay, Uruguay and South Brazil, it represents a more divergent germplasm pool than *Solanum tuberosum*, distributed in the Andes. This species is resistant to insects (Colorado potato beetle -*Leptinotarsa decemlineata*), fungi (common scab), bacterial wilt and soft rot, potato virus X and potato virus Y, root-knot nematodes and tolerate abiotic stress such as heat and drought.

S. chacoense Bitt. is for these reasons an important partner for potato (*Solanum tuberosum* L.) cultivars either in sexual crossings, when successful, or somatic hybridization experiments (Cheng & al., 1995 [3]). If somatic hybridization is applied, after electrofusion the culture of fusion products in the presence of antibiotics, which have no inhibitory effects on protoplast development, for preventing bacterial contamination may also prove very useful. Such an antibiotic with positive effects was reported to be cefotaxime that proved essential for cell division of mesophyll protoplasts of *Passiflora edulis* (d'Utra Vaz & al.,

1993 [4]). Cefotaxime and sometimes other antibiotics are also used in transformation experiments to control *Agrobacterium tumefaciens* overgrowth (Narendra Ram & Mohandas, 2003 [5]) In many instances side effects of such antibiotics on *in vitro* plant cultures were reported, both inhibition and stimulation of morphogenesis being observed [6, 7]. As for cefotaxime, a cephalosporin with a wide antibacterial spectrum, there are some reports showing stimulation of plant *in vitro* development and growth of roots, calluses, and somatic embryos [8, 9]. The first report on genetic transformation of *S. chacoense* by using either reporter *gfp* gene and the selectable *npt*II gene or *msh2* gene involved in mismatch repair of DNA were reported [10]. A better understanding of the cefotaxime effects on *in vitro* growth will also help in improving transformation technologies in this species.

Ethylene is a well-known inhibitor of potato growth *in vitro.* Previously it was demonstrated that ethylene suppression by inhibitors such as silver thiosulphate (STS) improves *in vitro* regeneration of potato tissues and protoplasts [11]. The effect of ethylene inhibitors on *in vitr*o cultures of the wild *Solanum* species was less investigated.

In the last years it was recognized that besides external contamination of *in vitro* cultures a latent internal bacterial contamination could often escape routine sterilization procedures. Internal contaminants can colonize intercellular spaces and the junctions between the cells with positive effects upon *in vitro* grown plants [12]. Bacteria of the genus *Pseudomonas*, *Bacillus* or *Agrobacterium* can stimulate the growth and differentiation of the plant cells *in vitro,* can reduce vitrification, improve acclimatization or induce resistance to some pathogens [13]. Moreover, bacteria can be the source of phytohormones like auxines and cytokinins thus affecting *in vitro* morphogenesis of infected plants [14, 15, 16]. Although such effects were previously observed the change of plant phenotype towards dwarfism was only reported in relation to *Agrobacterium rhizogenes* infected plants [17].

We present here the effect of cefotaxime and of the ethylene inhibitor silver thiosulphate on *in vitro* multiplication of *Solanum chacoense* Bitt. stem fragments. It is shown that cefotaxime stimulates *in vitro* growth of *S. chacoense* regardless of the concentration. The presence of silver thiosulphate in the culture media has a stimulatory effect mainly on leaf fresh weight that might be beneficial for mesophyll protoplast isolation. Cefotaxime was also used to eliminate internal bacterial contamination of an *in vitro* selected dwarf plant. The effect of endogenous contaminants on *in vitro* plant growth in relation with the possibility to select novel somaclones is also discussed.

Materials and methods

Plant material

Solanum chacoense Bitt. (accession no. 2095) true seeds were provided by Potato Research Institute Brasov, Romania. The seeds were sterilized by washing in 70% ethanol for 1 min followed by 7% (v/v) Domestos (commercial bleach which contains about 5% sodium hypochlorite) for 20 min then the seeds were rinsed three - four times with sterile water and were aseptically germinated on MS ½ culture medium (Murashige & Skoog, 1962 [18]), with or without 3% (w/v) activated charcoal. *In vitro* regenerated plants served as source of nodal stem explants, for direct regeneration of axillary buds. For the micropropagation of stock plants the apex or stem fragments, with two nodes, were transferred each month on RMB_5 medium (Menczel et al., 1981 [19]).

Culture conditions

In order to analyze the effect of cefotaxime on tissue culture, jars with screw lids provided with ten aeration vents were used as culture vessel. In each jar 20 nodal stem fragments of 1 cm length, without leaves, were inoculated on 70 ml of RMB_5 culture medium.

The antibiotic solution was sterilized by filtration and added to the medium after autoclavation. Since the most used concentration of cefotaxime is 250 mg l^{-1} beside that concentration two more values were chosen, one four times lower (62.5 mg l^{-1}) and one higher, 350 mg l^{-1}. The reason was to have different values not very closed to the original one, but the highest value was kept to 350 mg l^{-1}in order to avoid toxic effects on plat tissue. The antibiotic stock solution was kept in the freezer at -20 ^{o}C. In order to assess if long storage of stock solutions could affect cefotaxime stimulatory effects on *in vitro* growth of *S. chacoense,* different solutions stored in the freezer for 2, 3 or 3.5 weeks were comparatively analyzed by their effect on root growth.

The cultures were kept in a growth room at 21 ^{o}C, for 15 days with a 16 hours day length 75.6 μmol m^{-2} s^{-1} fluorescent light intensity. At the end of the culture period (15 days), both for controls and treated explants, comparative measurements were made: root number and total length (mm) per shoot, stem length (mm), number of leaves and leaf fresh weight. Statistical analysis included calculation of mean values, standard deviation (SD) and significance by using comparison of means (t test) applying MedCalc computer program.

Silver tiosulfate (STS) was added to the culture medium as a solution made up at 1:1 ratio from 12 mM $AgNO_3$ and 96 mM Na_2SO_3 x $5H_2O$ [11]. The stock solution was kept in the refrigerator, at 4 ^{o}C. Nodal stem fragments without leaves, 1 cm length were also used as initial explants. They were cultured for 10 days in 12 cm diameter Petri dishes in a growth room at 21 ^{o}C, 16 h photoperiod 75.6 μmol m^{-2} s^{-1} light intensity. 50 ml of solid RMB5 culture medium per each culture vessel and 15 to 30 explants in each Petri dish have been used. At the end of culture period stem and root mean length (mm) and leaf fresh weight were measured and statistically analyzed (as above).

Identification, analysis and treatment with cefotaxime of a bacterial infected dwarf somaclone

During repeated transfer *in vitro* on RMB5 medium (MS salts plus B5 vitamins) some shoots exhibited a contamination restricted to stem base. The white bacterial colonies were able to grow on RMB5 solid media only in close contact with the stem tissue and never developed a real contamination. All contaminated shoots have developed a dwarf phenotype with short stem and high anthocyanin content in both stem and leaves (purple color). In order to prove if bacterial contamination is the cause of dwarf phenotype the explants from such a somaclone were transferred on RMB5 media supplemented with 250 mg l^{-1} cefotaxime. The growth in the presence of cefotaxime was compared with control plants (with normal growth and bacteria free) and dwarf phenotype (contaminated with bacteria). The length of stem and root, leaf number and leaf fresh weight were measured and mean values, standard deviation and significance were calculated (as above). The dwarf somaclone was transferred *ex vitro* in pots containing sterilized soil, in the laboratory. To prevent dehydration the plants were covered with nylon foil for 3-4 weeks. From acclimatized plants new *in vitro* cultures were initiated, after standard sterilization (as described above), in order to analyze phenotype stability. All experiments were repeated three times.

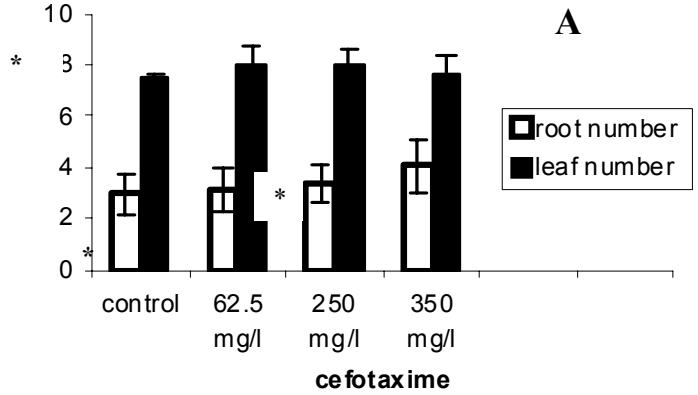
Results and discussions

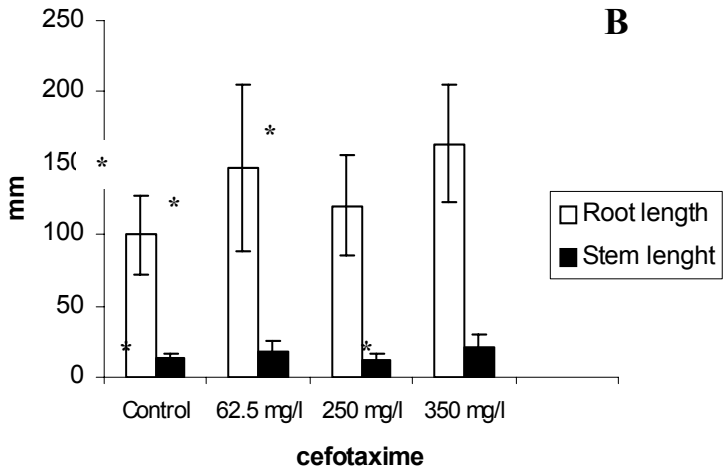
Cefotaxime stimulated *in vitro* development of *Solanum chacoense* Bitt. stem fragment-derived plantlets, at each concentration tested in our experiments. No direct correlation between the antibiotic concentration and its effect on plant growth was found (Fig. 1 A, B, C). The number of leaves was not affected by the addition of cefotaxime to the culture medium but the plantlets developed more roots with significant values for the two higher concentrations (250 mg l^{-1} and 350 mg l^{-1} - Fig. 1B). The total root length per plant and stem height were significantly stimulated at all tested concentrations but leaf fresh weight increased

significantly for the two lower concentrations of cefotaxime assessed in our experiments (Fig. 1). This kind of influence regardless concentration suggests that cefotaxime may act in a similar way to auxin-like hormones, although, the molecular structure of this antibiotic does not explain the production of breakdown components with such properties [20]. Nevertheless antibiotics were previously shown to stimulate somatic embryogenesis in the absence of growth regulators in several *Dianthus* cultivars [9].

Maintaining the antibiotic stock solution in the freezer for a longer period of time reduces the positive effects on plant growth. Thus, already 3 weeks old cefotaxime solution had an inhibitory influence on *S. chacoense* root growth (Fig. 2). These data suggest that when dissolved, the antibiotic is less stable even if it is maintained at -20 ^{o}C. For this reason preparation of fresh solutions of antibiotics is recommended for better repetability of the results.

Adding STS to the culture medium of *in vitro* cultures of *S. chacoense*, did stimulate only the growth of leaf, root and stem length, being not significantly different from the control explants (Fig. 3 A, B, C). The stimulatory effect of STS on leaf fresh weight might be important for healthy mesophyll protoplast isolation in somatic hybridization experiments. It was also shown that STS solution looses its effects after longer storage in the refrigerator, at 4 ^{o}C (data not shown). Previous studies on potato [11], demontrated that shoots, which were grown in the presence of STS in the culture media, yielded three times more protoplasts, than control shoots. The authors made two assumptions to explain these results. One assumption was that the ratio of leaf blade to stem tissue is much higher in the presence of STS, thus increasing mesophyll protoplast yield. However in our study no significant differences in leaf number were shown but there was an inverse ratio between leaf fresh weights and stem and root length. The other assumption was that leaf tissues originating from the shoots cultured in the presence of STS were more resistant to the stress induced by maceration enzymes.





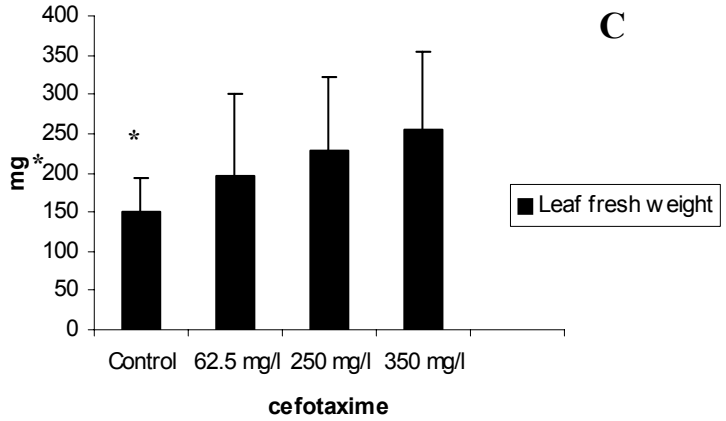


Fig. 1. The effect of three different concentrations of cefotaxime (62.5, 250 and 350 mg l^{-1}) on *in vitro* growth of *Solanum chacoense:* A) number of roots and leaves; B) total root lenght per shoot and stem length – mm; C) leaf fresh weight –mg (n = 15 – 20; bars = ± S.D; * significant at $p<0.05$).

Other authors [21] reported increase of ethylene biosynthesis during protoplast isolation by enzymatic digestion. Less ethylene was produced during potato protoplast isolation when the tissues were cultured in the presence of STS [11]. Our results suggest that the addition of STS to *in vitro* cultures of *Solanum chacoense* will be also benefic for mesophyll protoplast isolation in this species.

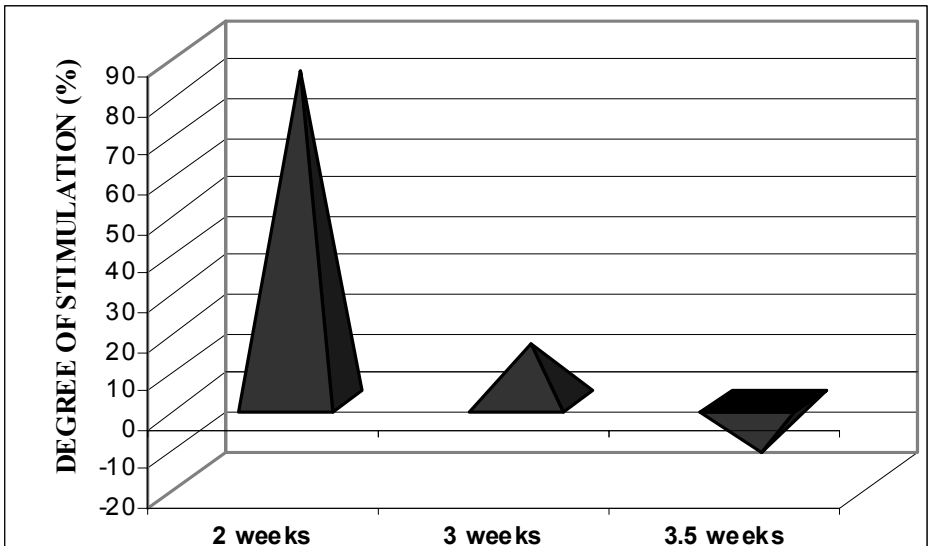


Fig. 2. Alteration of stimulatory effect of cefotaxime solution (250 mg l^{-1}) after storage at –20 ^{o}C for 2, 3 and 3.5 weeks as shown by its effect on *S. chacoense* root growth as percentage of stimulation.

A dwarf somaclone was observed during *in vitro* micropropagation of *Solanum chacoense*. The dwarf phenotype with short stem and high level of anthocyanins in the stem and leaves, as a consequence having a purple color (Fig. 4), was always associated with bacterial contamination, either at the base of the explants or internally. Bacteria having bacillary form were observed under the microscope at the base of contaminated explants as well as in the cellular sap when shoots were grinded with mortar and pestle. All attempts to culture and isolate the bacteria failed (data not shown). The bacteria were able to proliferate only in the presence of plant tissue behaving as an endoparasite. All dwarf shoots treated with cefotaxime reverted to an apparently normal growth but they developed stems only half as

long as the stems of control, uninfected plants (Fig.4, and Fig. 5 A, B, C). Although dwarf shoots have developed very short roots, cefotaxime treatment induced almost normal growth of the roots. Leaf number was better after cefotaxime treatment of the dwarf somaclone but still lower as compared to control plants but leaf fresh weight reached the same value as the control shoots after cefotaxime treatment (Fig. 5). The dwarf somaclone acclimatized and grown in pots in the laboratory developed a great number of stolons, which lately produced a bushy phenotype, which we thought might by exploited to produce a new ornamental pot plant (Fig. 6). Upon retransfer *in vitro* only part of the plants maintained the dwarf phenotype, those still contaminated at least internally by the not yet identified bacillus, proving that the bacterial contamination is the cause of dwarf phenotype.

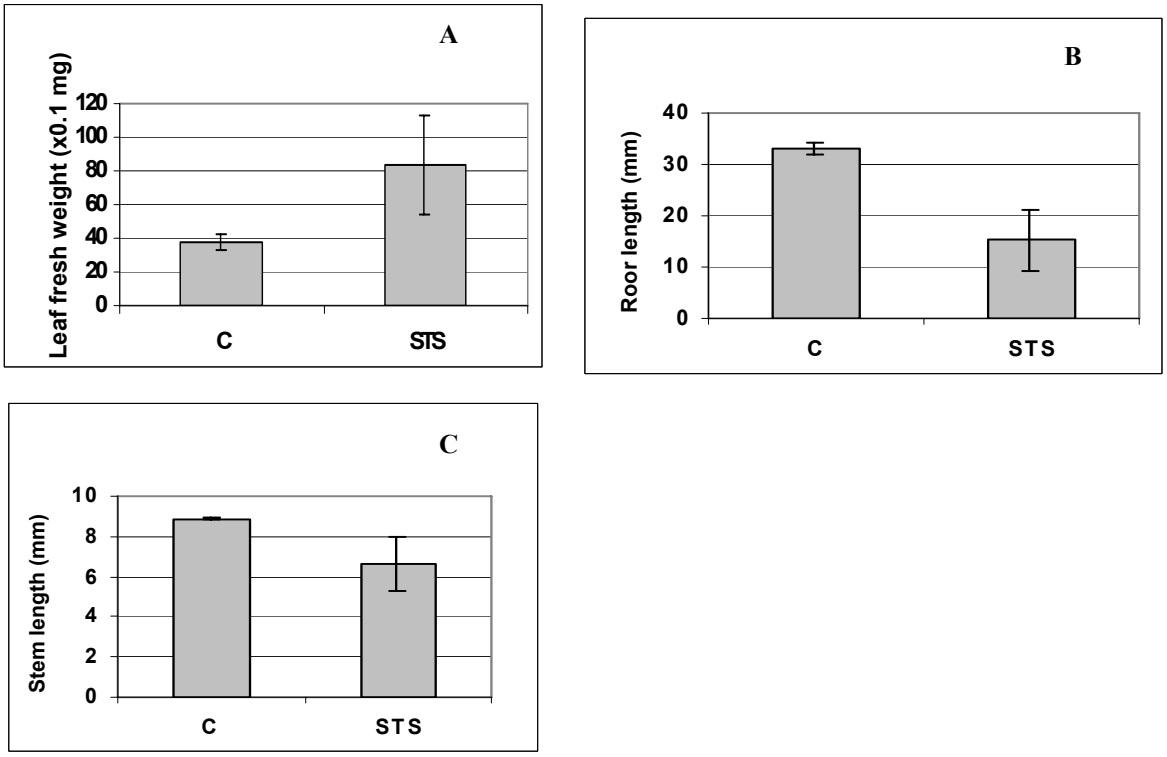


Fig. 3. The effect of silver thiosulohate (STS) on *in vitro* growth of *Solanum chacoense* in comparison with control shoots (C): A) mean leaf fresh weight x 0.1 mg; B) mean root length in mm; C) mean stem length (mm). (n=15-30; Bars = ± SD; * significant at $p<0.05$).

Fig. 4. *Solanum chacoense* dwarf somaclone maintained *in vitro* (1) in comparison to wild type (3) or cefotaxime treated ex-dwarf plants (2) after 3 weeks in culture on RMB5 medium.

The presence of cefotaxime in the culture medium was able to reduce the inhibitory effect of bacterial contamination upon *Solanum chacoense in vitro* growth because it can penetrate dip into the plant tissue and exerts its antibacterial action.

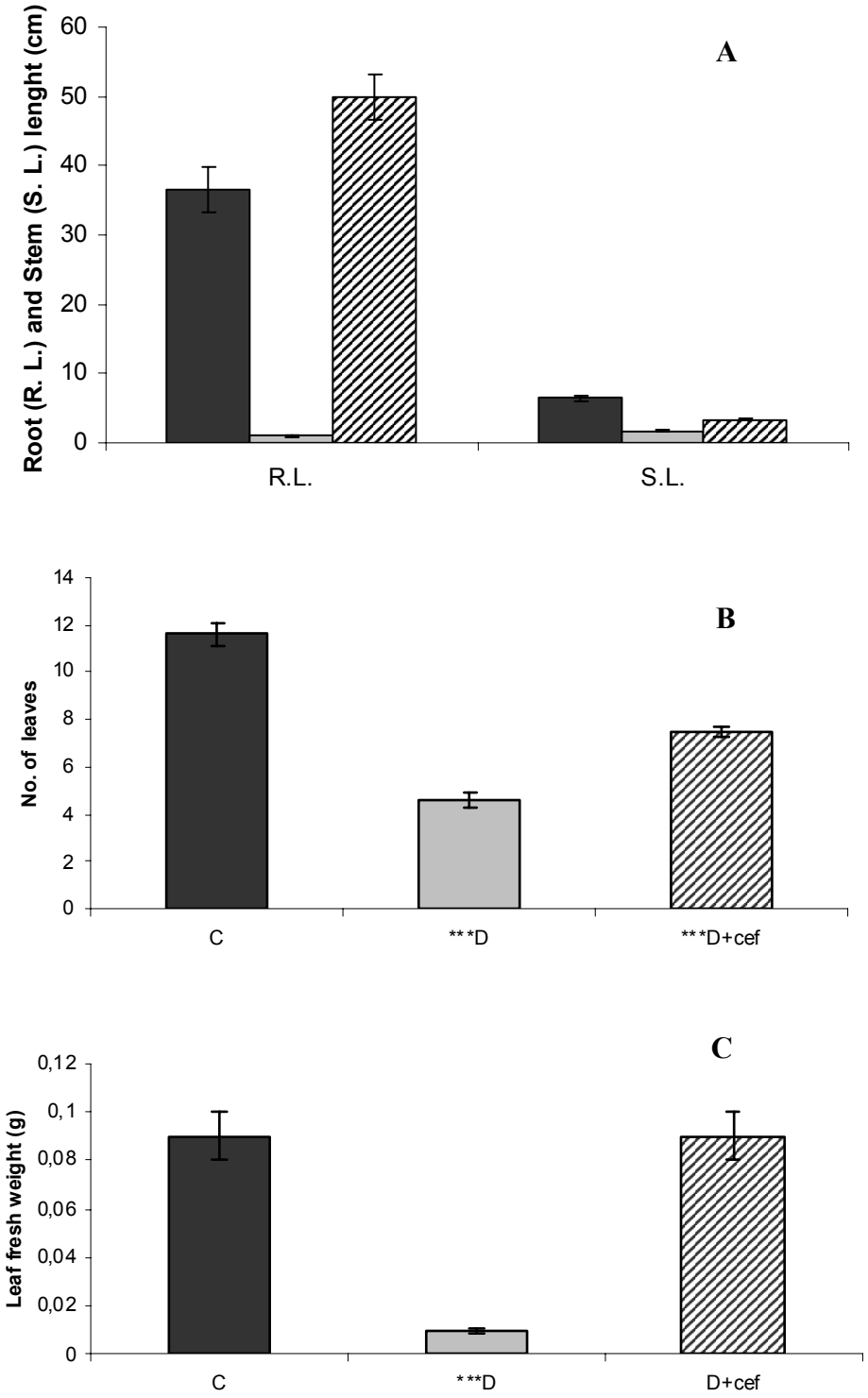


Fig. 5. The effects of cefotaxime (D+cef) on dwarf *Solanum chacoense* somaclone (D) growth in comparison with wild type plants (C): A) total root length (RL) and stem length (SL) – in cm; B) number of leaves; C) mean leaf fresh weight – g (n= 15 – 20; bar= ± S.D, *** significant at $p \geq 0.001$).

Fig. 6. *Solanum chacoense* dwarf somaclone transferred *ex vitro* shows development of many stolons (arrowed), which formed when planted in pots in the laboratory a bushy phenotype (bar = 1 cm).

Microscopic observations confirmed the absence of bacteria from ex-dwarf cefotaxime treated shoots. A routine sterilization is not enough to remove such contaminants [12]. Previously it was shown that bacteria can produce different phytohormones like cytokinines and the stimulatory effect of cytokinines on anthocyanin synthesis was demonstrated [22]. If bacteria produce such phytohormones they can act as signals with a complex effect on plant physiology, affecting both plant growth and development and the interrelationship between bacteria and plant tissue [20]. Indirectly the antibiotics as cefotaxime may stimulate *in vitro* differentiation and organogenesis, even in elite genotypes, as recently demonstrated in different laboratories [23, 24, 25]. Such an interrelationship between bacteria and/or antibiotics and plants *in vitro* needs to be further investigated. Understanding the nature of this interaction might be very useful for controlling dwarf phenotype of *S. chacoense* stability *in vitro* and *ex vitro* and might be exploited for the production of a new ornamental pot plant.

Conclusions

Cefotaxime can be used as an antibiotic both to control bacterial contaminants *in vitro* or *Agrobacterium* overgrowth in transformation experiments, since it has benefic effects on *in vitro* growth of *S. chacoense* and on other species. STS is benefic for leaf growth and is recommended for the improvement of mesophyll protoplast isolation in this species. A dwarf somaclone induced *in vitro* in *S. chacoense* might prove useful as a novel ornamental plant.

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