CEDR Transnational Road Research Programme
Call 2016: Invasive Species and Biodiversity

funded by Germany, Sweden, Netherlands, Ireland, Austria, Slovenia and Norway

CEDR
Conférence Européenne des Directeurs des Routes
Conference of European Directors of Roads

ControllnRoad
Controlling the spread of invasive species with innovative methods in road construction and maintenance

Greenhouse assays

D3.2
July, 2019

controllnroad

CEDR
Conférence Européenne des Directeurs des Routes
Conference of European Directors of Roads

CEDR Call 2016: [Invasive Species and Biodiversity] ControlInRoad
Controlling the spread of invasive species with innovative methods in road construction and maintenance

Report on the greenhouse assays performed

Planned delivery date: 01/07/2019
Actual delivery date: 01/07/2019

Start date of project: 01/09/2017

Authors of this deliverable:
Friederike Trognitz, AIT Austrian Institute of Technology, Austria
Theresa Ringwald, AIT Austrian Institute of Technology, Austria
Lisa Lutz, AIT Austrian Institute of Technology, Austria
Swen Follak, AGES, Austria
Angela Sessitsch, AIT Austrian Institute of Technology, Austria

PEB Project contact: Pia Bartels

Version: 1 [06.2019]

Table of contents

Executive summary

Biological control on invasive alien species on road sides is still an underexploited measure to manage invasive alien plant species (IAPs). The use of herbicides is not in every country allowed and with the discontinuation of herbicides their use is getting more and more restricted. CSIRO in Australia stated that biocontrol is the most cost-effective and environmentally benign solution for managing IAS and native enemies (https://www.csiro.au/en/Research/BF/Areas/Invasive-species-and-diseases/Biological-control). In Europe, only little research has been conducted in this field. The reasons are that herbicides have been still the most effective and economical way to control unwanted vegetation. The use of biocontrol solutions against plant pathogens is meanwhile widely accepted and represents an emerging market, but research on weed control with biological agents is still underexplored.

IAPs on roads have space to establish and spread if the native vegetation is reduced. To control IAPs, either the plant itself has to be controlled or the growth of the native vegetation has to be promoted. Within the framework of this project, several avenues of biological control based on the use of plant-associated microorganisms have been tested regarding their potential to control IAPs. These are the following:

- Strengthening of native seed mixtures used for greening road verges
- Germination inhibition of ragweed
- Selecting *Festuca rubra* varieties with high allelopathic effects
- Isolating and testing bacterial strains for the control of Himalayan balsam
- Treatments for safe disposal of the rhizomes from Japanese knotweed

Plant growth-promoting bacteria (PGPB) were applied on native seed mixtures used for greening road verges to outcompete specific IAPs. Several bacterial strains isolated in previous projects were tested. Unfortunately, none of the tested strains promoted the growth of the plants in the seed mixtures tested, although the selected bacteria had shown plant growth promotion on wheat. A more thorough screening of microorganisms, ideally isolated from the target plants, would be required to identify candidates for the further development of a treatment enhancing growth of native plant species competing with IAPs.

The second approach was to test plant-associated bacteria isolated from ragweed for their capacity to inhibit germination of ragweed in the presence of different seed mixtures. The strains were selected based on results regarding germination inhibition from a previous project. The germination rates of ragweed were different in the presence of two seed mixtures, which were tested in frame of this project. Furthermore, results varied between trials. In the first trial conducted in March none of the bacterial strains had an effect on germination. In the second trial in May, one strain in the seed mixture B3 promoted germination significantly. Generally, variable results are frequently obtained when working with biological organisms requiring more rigorous testing and development of application procedures enabling more constant results.

F. rubra is known for the allelopathic effect against broadleaved plants. To find out, if the high content of *Festuca rubra* in the seed mixtures affect germination of ragweed, three different varieties of *F. rubra* were tested together with ragweed seeds. The variety Raisa significantly reduced the germination of ragweed. Also, the variety Melissa reduced the germination, but the effect was not significant. This finding can already be used to out-compete ragweed during road construction by using seed mixtures with a high content of *F. rubra* varieties with high allelopathic effects such as the variety Raisa. In the field test 2019 one treatment was planned for using *F. rubra* variety Raisa, but because of the dry summer in 2018, no seeds were

available. Therefore, all three varieties were mixed together and used in the field experiment against ragweed.

In frame of the project the development of biological control agents against Himalayan balsam was started. More than 200 bacterial strains were isolated from Himalayan balsam roots and shoots of plants collected in the Danube river site in Tulln in 2018. The strains were tested on lettuce because collected Himalayan balsam seeds did not germinate in spring 2019. Several strains showed a germination inhibition in several experiments. The strains still need to be validated on Himalayan balsam seeds and plants and more rigorous testing is required as well as the development of application practices. Nevertheless, here the aim was to test the potential of such an approach.

To support the safe disposal of the rhizomes from Japanese knotweed, several heat treatments were performed. The heat treatment at 121°C for 20 min prevented the re-sprouting of the rhizomes, whereas lower temperature did not. The soil-free storage has to be evaluated in the next vegetation period and also if a storage period of one year without soil prevents re-sprouting.

1 Introduction

Invasive alien plants (IAPs) are a major problem on disturbed soils on which the development of endemic, native plants and seeds is disadvantaged. Such disturbed soils can result from road construction works that create adverse conditions on patches, such as verges, slopes and embankments. IAPs may have an advantage on these disturbed soils because they are specifically adapted or because IAP seed and propagules may be distributed by road construction activities and materials used.

Plant growth promotion by plant-associated microorganisms

The greening of the newly constructed road verges is commonly initiated by sowing seed mixtures of plant species which are deemed suitable for the specific location. This initial greening often has to be done under unfavourable conditions due to shortness of time and resources. Under these circumstances, the use of specific plant growth-promoting bacteria (PGPB) in seed coatings and other appropriate forms of application can be very useful to support the establishment of the plants. PGPB may strengthen the emerging seed and young seedlings by accelerating their germination and growth. This positive effect of PGPB is already utilized in agriculture for the reduction of the use of chemical substances in cropping systems (O'Callaghan 2016). Several products for seed treatment and seed coating with PGPB are available on the market (for example Proradix from Sourcon Padena or Rhizovital from Abitep).

Some plant growth-promoting bacteria can directly protect plants against various forms of abiotic and biotic threats (Brader et al. 2017, Mitter et al. 2016), some can antagonize soil-borne pathogens (Toumatia et al. 2016, Haas & Defago 2005), others may improve the nutrition of their host plants (Pfeiffer et al. 2017, Mantelin & Touraine 2004, Hayat et al. 2010) and yet others have been found to directly promote the plant's growth (Lugtenberg & Kamilova 2009, Ahemad & Kibret 2014). Little research has so far been directed toward the potential application of PGPB for the efficient greening of public spaces and roadsides. More has been done in the much larger agricultural market sector, but only few results from agricultural research can be transferred directly to the area of public works and road construction. However, with the understanding of how PGPB act it may be possible to adapt this technology to the utilization of plant-associated bacteria for the proper greening of roadsides.

Bioherbicides based on plant-associated microorganisms

Specific plant-associated bacteria can produce deleterious effects on their hosts when they are applied at high concentrations. Such agents can be used for the biological control against undesired plants. The classical concept of biological control is based on microbes that occur in the natural habitats of invasive alien plants and that repress the growth of these plants in their original habitat. These organisms are introduced in regions were the plant has no native enemies. In contrast to this classical biological control, the "inundative" biological control approach utilizes a wide range of vectors and mechanisms to bring about biological control. This frequently employs methods of repeatedly applying specific microbes at high concentrations when the pest or weed causes a problem, in analogy to the use of a pesticide. The microbes are isolated from the IAP at the location that it has invaded. These "inundative" biocontrol agents can be based on bacteria, fungi, viruses or insects. The biological agent is not expected to be self-sustaining (Boyetchko 1997, Trognitz et al. 2016). The applications and the resulting control effect are usually transient and sometimes, several subsequent

applications have to be made (Bale et al. 2008). In contrast to chemical herbicides, microorganisms can also damage seeds. Several bioherbicides have been developed in the past years, however, no commercial product is on the market in Europe. Most products have been developed for use in agriculture and very rarely for the control of IAPs. One good example of the application of an endemic fungus is the use of *Chondrosteum purpureum* in the control of black cherry (*Prunus serotina,* de Jong 2000). Fungal mycelium is applied onto fresh wounds and cuttings in the wood. With this method, black cherry could be efficiently controlled in the Netherlands. This fungus was also tested on other trees including *Betula pendula*, *Alnus incana*, *Populus tremula*, *Salix caprea*, *Acer negundo*, *Hippophae rhamnoides* and *Robinia pseudoacacia* (Lygis et al. 2012). The control of the trees was similar to chemical herbicides except for *R. pseudoacacia* and *H. rhamnoides*. In another study, *C. purpureum* was used in the control of red alder from North America (*Alnus rubra*, Becker et al. 2006). In the first year, 92% of the red alder died and in the second year 100% of the trees did not re-sprout. The fungus can get access only via open wounds. It was not found on neighbouring trees, which makes it a particularly promising bioherbicide for the control of selected trees.

Since 2016 *Impatiens glandulifera* is listed in the EU regulation 1143/2014. Control measures have to be developed to mitigate this plant and reduce the spread. Currently contaminated sites are mostly mowed or mulched. The use of herbicides is mostly prohibited because of the neighbouring water resources or destruction of the native vegetation, which makes the site prune for new infestation. Therefore, a measure targeting only *I. glandulifera* without any negative effect to the environment is needed. In our study our aim was to isolate and screen bacteria isolated from *I. glanduliera* and test if the strain inhibits the germination of *I. glandulifera*.

The unique potential of biological agents to control the growth of specific weeds makes them suitable for the management of IAPs. However, intensive work is needed to find the right control agents under different environmental conditions. Beside the biocontrol effect also non-target effects have to be carefully evaluated.

Plant allelopathic effects by Festuca rubra

Several plant species produce exudates, which are phytotoxic to neighbouring plants, thereby creating an advantage in the competition for nutrients, light and water (Duke 2007). Some of these products are potent toxins, such as juglone from black walnut and sorgoleone from sorghum. *Festuca rubra commutata*, a representative of eight out of 80 subspecies of fescue (Bertin et al., 2007; Tworkoski & Glenn, 2012), produces the potent phytotoxin meta-tyrosine in high concentration. This non-protein amino acid has been proposed as a natural herbicide (Bertin et al. 2007) as it has been shown to strongly suppress the growth of weeds (Bertin et al., 2007; Tworkoski & Glenn, 2012). The level of suppression of broadleaved weeds by fescue and ryegrass was similar to that of chemical herbicides (Tworkoski & Glenn, 2012).

Disposal of IAPs

Cut or excavated material from sites infested with IAPs has to be treated as hazard waste and for each IAP different treatments are needed. For *Fallopia japonica* special care is taken, because already root parts of only 0.7 g are able to regenerate and form new plants (Adachi et al 1996, Fuchs et al. 2018). For a safe storage of excavated material, the rhizomes should be buried at a depth of 2 to 5 m depth and covered with geotex material. The method is in regard to air pollution environmentally friendly but expensive and leads to drainage problems.

In the frame of ControlInRoad, strategies and agents, alternative to chemical pesticides, were tested for the control of IAPs, with the objective to possibly develop methods of biological control that can be suggested for use by the road authorities. These tests included:

1) Application of several different strains of plant growth-promoting bacteria (PGPB) to commercial mixtures of seeds that are commonly used in the technical greening of newly constructed roadsides and to test the intrinsic potential of these PGPB to boost seed germination and the seedlings' tolerance to drought stress.
2) Tests of selected PGPB in combination with grass seed mixtures, for their potential to reduce the germination of ragweed (*Ambrosia artemisiifolia*).
3) Tests with different *Festuca rubra* varieties for their ability to reduce the germination of ragweed (*A. artemisiifolia*)
4) Isolation of plant-associated bacteria from Himalayan balsam (*Impatiens glandulifera*) and their application to reduce seed germination and plant growth of this invasive neophyte plant.
5) Heat treatment of Japanese knotweed (*Fallopia japonica*) rhizomes to inhibit re-growth.

2 Materials and Methods

2.1 Description of commercial seed mixtures

Two different native seed mixtures (commercial blends of seed), recommended and frequently used for slope greening, were selected for greenhouse tests: 1) mixture for below 1000 m sea level, sunny and summer-dry habitat (Table 1) and 2) mixture for below 1000 m sea level, fresh and not necessarily summer-dry habitat (Table 2). The seeds were provided by Kärntner Saatbau. The ratio of grass vs. clover seeds within each of the two commercial seed mixtures was nearly identical (78:22 in B3 and 80:20 in B4), but the species composition of both groups, grasses and clover, varied in the two mixtures as can be seen in Tables 1 and 2.

Table 1: Species composition of the commercial seed mixture B3.

ReNatura® Böschung B3 und B3 Mantelsaat® (MS)			
Anwendungsbereich:	Zur Böschungsbegrünung in Seehöhen bis 1000 m, bevorzugt auf sonnigen und sommertrockenen Standorten.		
Aussaatmenge:	75 - 100 kg/ha	Packungsgröße: 25 kg	Art.-Nr. B3: 21055 Art.-Nr. B3 MS: 21057
Anteil	**Art**		**Typ**
9,00 %	Aufrechte Trespe	*Bromus erectus*	Ökotyp A
3,00 %	Glatthafer	*Arrhenatherum elatius*	
5,00 %	Knaulgras	*Dactylis glomerata*	
9,00 %	Schafschwingel	*Festuca ovina*	
10,00 %	Furchenschwingel	*Festuca rupicola*	Ökotyp A
29,00 %	Rotschwingel	*Festuca rubra rubra*	
5,00 %	Rotstraußgras	*Agrostis capillaris*	
3,00 %	Schmalblättrige Rispe	*Poa angustifolia*	Ökotyp A
5,00 %	Englisches Raygras	*Lolium perenne*	
4,00 %	Weißklee	*Trifolium repens*	
2,00 %	Hornklee	*Lotus corniculatus*	
6,00 %	Luzerne	*Medicago sativa*	
10,00 %	Esparsette	*Onobrychis viciifolia*	

Table 2: Species composition of the commercial seed mixture B4.

ReNatura® Böschung B4 und B4 Mantelsaat® (MS)			
Anwendungsbereich:	Zur Böschungsbegrünung in Seehöhen bis 1000 m, bevorzugt auf frischen und nicht ausgesprochen sommertrockenen Standorten		
Aussaatmenge:	75 - 100 kg/ha	Packungsgröße: 25 kg	Art.-Nr. B4: 21063 Art.-Nr. B4 MS: 21064
Anteil	**Art**		**Typ**
10,00 %	Glatthafer	*Arrhenatherum elatius*	
6,00 %	Knaulgras	*Dactylis glomerata*	
35,00 %	Rotschwingel	*Festuca rubra rubra*	
6,00 %	Rotstraußgras	*Agrostis capillaris*	
10,00 %	Wiesenlieschgras	*Phleum pratense*	
5,00 %	Englisches Raygras	*Lolium perenne*	
4,00 %	Wiesenfuchsschwanz	*Alopecurus pratensis*	
4,00 %	Weißes Straußgras	*Agrostis alba*	
6,00 %	Hornklee	*Lotus corniculatus*	
12,00 %	Weißklee	*Trifolium repens*	
2,00 %	Schwedenklee	*Trifolium hybridum*	

2.2 Bacterial strains and seed inoculation

2.2.1 Bacterial strains for plant growth promotion

Three different bacterial strains were selected. The strains were isolated from surface-sterilized wheat seeds and had shown growth promotion effects on wheat in previous experiments (own results). The three isolates are 4-3 (*Pseudomonas* sp.), 5-4 (*Kocuria* sp.) and 5-8 (*Pantoea* sp.)

The bacterial strains were grown overnight in 10% TSB. The OD_{600} was adjusted for each treatment to a value of 0.2 using 1x PBS-buffer (Phosphate-Buffered Saline) as blank. The seeds of both native seed mixtures were inoculated with one of the three strains suspended in 1x PBS-buffer (OD_{600} 0.2) or with 1x PBS-buffer devoid of bacteria (as a negative control).

The inoculated seeds were sown in 3 L pots filled with standard potting soil (Einheitserde SP ED63 T and Null Erde CL SM 0 without any addition of fertilizer, http://www.einheitserde.de/produkte/topfsubstrate/). Based on the recommendation of the seed producer, 0.25 g seeds were placed on the top of the soil. The pots were kept in the greenhouse and watered regularly. By day 15 after sowing, the watering was suspended, to expose the seedlings to increasing drought stress simulating a dry summer period. Watering was resumed after another 12 days. Aboveground (green) plant tissues were harvested five weeks (36 days) after sowing and dried for 3 days at 105°C before the dry green biomass was weighted.

For analysis, multiple t-tests and ANOVA implemented in R (https://www.r-project.org/) were applied using a confidence interval of 0.95.

2.2.2 *Bacterial strains for the control of ragweed germination and application to ragweed*

Bacterial strains

Six bacteria with the proven, strong ability to interfere with the development of ragweed seedlings were selected (Table 3). These six strains had been discovered in a previous project of our research group (Project No. LS12-016, NFB NÖ Forschung und Bildung). In previous experiments we could show that each strain individually reduced the germination rate of ragweed seed by up to 30% in the greenhouse. For this project overnight cultures (in 10% TSB liquid medium) were adjusted with 1x PBS buffer to suspensions containing 10^8 colony forming units (cfu)/ml.

Table 3: Bacterial strains used for testing the control of ragweed germination

Strain name	Genus
473	*Rhizobium* sp.
6	*Acidovarox* sp.
660	*Pseudomonas* sp.
431	*Arthrobacter* sp.
444	*Microbacterium* sp.
504	*Bacillus* sp.

Application of the bacteria and plant experiment

The humidified ragweed seeds were stratified for optimal germination at 4°C during ten days on wet filter paper in the refrigerator. After stratification, the ragweed seeds were placed on the surface of standard potting substrate (“Gärtnerische Einheitserde”, see above, section 2.1) in pots, and 15 ragweed seeds were sown in three replicates per treatment. Then the seed mixtures B3 or B4 (Tables 1 and 2, above) (0.25 g each) were added immediately. Directly after sowing, 100 ml bacterial solution (10^8 cfu/ml in 1x PBS) were added to each pot. For the negative controls the same volume of 1x PBS buffer was used. The pots were placed on benches in a greenhouse in a randomized order and watered daily. After 11 days the bacterial treatment was repeated once.

The experiment was performed twice, one replication in March 2018 and one in May 2018. After 42 days in the first trial, and after 27 days in the second trial, the numbers of emerged ragweed seedlings were counted.

2.2.3 *Investigation of the inhibitory abilities of red fescue (*Festuca rubra*) on the germination of ragweed*

Three varieties of red fescue, Melitta, Smaragd and Raisa, provided by Hesa Saaten (Himberg, Austria), were used to test their potential inhibitory effect on the germination of ragweed. *Festuca rubra* has been described to produce inhibitory root exudates (Bertin et al. 2007). The ragweed seed was stratified at 4°C during 7 days on wet paper in the dark, as described above (section 2.2.2) and 10 seeds of ragweed together with 20 seeds of red fescue were placed in a pot containing standard potting substrate (“Einheitserde” SP ED63 T, see above). For every

fescue variety-ragweed combination, three replicates of 10 seeds with the corresponding red fescue variety were laid out in a completely randomized block design in the greenhouse. The germination of ragweed and *Festuca* was rated every 5 days.

2.3 Investigations on the biocontrol of Himalayan balsam (Impatiens glandulifera)

2.3.1 Isolation of plant-associated bacteria from I. glandulifera

To obtain potential biocontrol bacterial strains against *I. glandulifera* plant samples were collected at five sites close to the town of Tulln, Austria. Root and shoot parts were used for the isolation.

Samples of fresh plant tissue of approximately 100 g fresh weight were ground in a sterile mortar with the addition of 4 ml sterile 0.85% (w/v) sodium chloride. One hundred µl of the resulting suspension were diluted with 0.9% NaCl to obtain a dilution series of one part in 0 (undiluted), 10, 100, 1000, 10000, and 100000. From each dilution 100 µl were spread onto sterile TSA plates (Difco) and incubated at 27°C for 5 to 7 days. Emerging single colonies were picked from these plates and transferred for single strain isolation to individual plates containing solidified TSA medium. After one to four days of incubation at 27°C, plates containing well-grown bacterial colonies were sealed with parafilm and stored at 4°C until further use. For the molecular taxonomic determination of these bacteria species, the DNA sequence of their partial 16S rRNA genes were obtained and subjected to comparative searches within the Bacterial Ribosomal Gene Database at Michigan State University, USA (RPD), as follows. DNA was isolated from the bacteria using the nexttec kit according to the manual provided by the manufacturer (nexttec Biotechnologie GmbH, Leverkusen, Germany). For the amplification of a specific 16S rRNA gene regions within these DNA samples, the forward PCR primer 8F 5' AGAGTTTGATCCTGGCTCAG 3' and the reverse primer 926R 5' CCGTCAAATTCCTTTRAGTTT 3' were applied. Each PCR reaction contained 2 µl template DNA (20-150 ng) mixed with 1x BD buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer and 1 U Firepol® polymerase (Solis BioDyne), complemented with sterile water to a 20 µl volume. Amplification was performed with a Biometra thermocycler (Biometra, Germany) following the cycling protocol of: initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 30 sec, primer annealing at 53°C for 45 sec and elongation at 72°C for 90 sec, finished by a final elongation step of 10 min at 72°C. For determination of the individual 16S rRNA gene sequences, the amplicons from our PCR reactions were sent to the service at the LGC® Genomics GmbH (Berlin, Germany) for one-direction sequencing starting at the single primer 8F. The obtained sequences were digitally trimmed and searched for similarity against the ribosomal database project RDP http://rdp.cme.msu.edu/.

For the investigations described in 2.4.2. through 2.4.7., we chose from the above isolated strains those that were determined as *Pseudomonas* spp. and *Microbacterium* spp. because these genera have shown potential bioherbicidal activity in other studies.

2.3.2 Seed germination-inhibitory properties of the isolated bacteria

Seeds of *I. glandulifera* were collected in summer 2018 in Tulln. The seeds were stored under dry conditions before covering them for 4 weeks (in Winter 2018/19) with humid soil in a yard

to mimic the natural overwintering. Unfortunately, these seeds did not yet germinate in February. Our agenda of the project, however, offered only time for testing the inhibitory properties on seed germination of the isolated bacterial strains in February-March 2019. Therefore, we carried out the germination tests on lettuce seed (*Lactuca sativa*, open pollinated variety Maikönig). Lettuce is a widely used model plant in phytotoxicity tests.

For testing the bacterial isolates (55 *Pseudomonas* spp. strains and 25 *Microbacterium* spp. strains), sterile vermiculite pearls in a Petri dish were wetted with 10 ml autoclaved water. A filter paper disc was placed on top of the vermiculite and 20 surface sterilized lettuce seeds were distributed. For the inoculation with the bacterial isolates, 10 ml of a 10^5 cfu (colony forming units)/ml overnight liquid culture were added to the seeds. Every 2-3 days the moisture was controlled and the number of germinated seeds recorded. Each treatment level consisted of three Petri dishes.

For statistical analysis, multiple t-tests and ANOVA in R (https://www.r-project.org/) with a confidence interval of 0.95 were applied.

2.3.3 Dose-dependent effects of seed germination inhibition

Overnight liquid cultures of bacteria were adjusted to a concentration of 10^8 cfu/ml. The overnight culture with the adjusted concentration was centrifuged at 4500 g (at 4°C) for 10 minutes. The bacterial pellet was re-suspended in 0.9% NaCl. A dilution series in tenfold steps, starting from 10^8 down to 10^2 cfu/ml, was prepared. Around 15 surface-sterilized lettuce seeds were incubated, imbibed in the individual dilution level at room temperature for one hour. As a control, 15 seeds were incubated in 0.9% NaCl solution. Subsequently, three seeds from each dilution level and the control devoid of bacteria were put in a 24-well microtiter plate on top of a layer of 1.5% water-agar. For each strain, three replicates of three seeds were prepared. For analysis, the length of the emerging roots and shoots was measured with a ruler after one week of germination.

The data were analysed using the multiple t-test procedure in R (https://www.r-project.org/).

2.3.4 Auxin production by the isolated bacteria

Auxins are a group of natural (indole-3 acetic acid, IAA) and synthetic (examples, 2,4-dichlorphenoxy acetic acid; 2-4 D, indole butyric acid; IBA) plant growth hormones and can exert plant growth-promoting as well as -inhibitory effects, depending on the concentration of the hormone and other conditions. This type of hormone has been used in synthetic growth and weed control preparations (examples, ‘Agent Orange’, ‘Seradix’ rooting powder).

Some bacteria are able to produce auxin in different concentrations. To test if the strains produce auxin *in vitro* the Salkowski assay (Sarwar et al. 1992) was used. The strains were grown in 50% TSA medium at 28°C for 24 hours. The colonies were transferred to YMA medium with and without tryptophan and grown for 96 hours at 28°C in the dark. The cultures were centrifuged for 10 minutes at 4500 g in a 4°C pre-cooled centrifuge. Triplicates of 120 µl of the cell-free supernatant were transferred to a U-bottom shaped microtiter plate. To each well of the microtiter plate 80 µl of the Salkowski reagent was added and incubated for 30 min in the dark. For the standard curve a serial dilution of 0.01% IAA was included in the assay

starting from 200 ml/µl down to 5 ml/µl IAA. The plate was measured at 530 nm extinction in a plate reader (Synergy MX, BioTek, Germany)

2.3.5 Growth inhibition of seed and plants of I. glandulifera

Tests with *I. glandulifera* seeds

The collected seeds from 2018 started slowly to germinate in May 2019. To test if the *I. glandulifera* seeds can be used for the host assay we started with two bacterial strains (IG-R-60 and IG-R-138.3A). All tests were performed in the greenhouse of AIT in pots using a soil mixture of two parts potting soil and one part quartz sand. Ten seeds of *I. glandulifera* were sown per pot. Per bacterial strain 10 pots were prepared. The seeds of this species germinate and shoot in the dark and therefore, we placed the seed 1-2 cm beneath the surface of the moist substrate. The bacteria were cultured overnight for propagation in 10% TSB medium (Sigma Aldrich, Germany) at 26°C and 160 rpm in a 2 L flask. The bacterial cells were harvested via centrifugation for 10 min at 4500 g and 4°C. The pellet was re-suspended in 0.9 % NaCl. The OD_{600} (optical density) was measured to determine the concentration of the bacteria and the suspension to use was adjusted to an OD at 600 nm (OD_{600}) corresponding to a value of 10^5 cfu/ml. Forty ml of this bacterial suspension was added to each pot containing the plant seeds. The pots were watered regularly. As a control, 0.9% NaCl solution was added to the pots of the control treatment, at the same quantity as the bacterial suspension (40 ml). The number of germinated seeds and the length of the seedlings were measured after 4 weeks. Because the germination still was very low (below 10%), no other strains were tested. Therefore, the strains were tested on juvenile *I. glandulifera* plants.

Tests with *I. glandulifera* plants

Juvenile seedlings of Indian balsam were dug out at a site near the banks of the Danube river close to the natural swimming pool “Aubad” at the town of Tulln and were transferred to 100 L pots filled with the natural soil from the sites. The plants were kept in the wirehouse of AIT and watered frequently to keep the plants moistened. Two growth stages of *I. glandulifera* plants were chosen for the experiments; a younger stage when the plants had reached an average length of 30 cm and a diameter at the stem base of about 1 cm and 4-6 leaves at an advanced stage when the plants were 80 cm tall and had 1-2 cm stem diameter (fully grown, prior to flowering stage). All plants were decapitated by removing the upper half (young growth stage) or the top above the main branching (adult stage) of the stems with a pair of secateurs.

For all treatments, 4 to 5 plants per pot and 5 pots (replications) were used. The bacterial suspension was sprayed with a hand-held sprayer until run-off (maximum 50 ml of a 10^8 cfu/ml suspension) directly onto the surface of the fresh cuts and also onto the remaining stems and leaves. Fresh liquid 10% TSB medium devoid of bacteria was sprayed on the control plants cut in the same way as the plants that received bacteria.

2.3.6 Testing the best performing bacterial strains on non-target plants

To test if the selected bacteria only have a deleterious effect on *I. glandulifera* and not on other *Impatiens* species, we performed the concentration assay as described in section 2.3.3. For this assay *Impatiens walleriana* (Busy Lizzie), a widely used ornamental plant, was used. Seed

were purchased from Austrosaat (Vienna, Austria) and tests were performed as described above.

2.4 Search for methods of preventing the regrowth of rhizomes of plant Fallopia japonica

Rhizomes of Japanese knotweed were collected on an infested site in Schützen am Gebirge, Burgenland, Austria, where this invasive plant has established itself several years ago.

Three independently collected rhizomes were used in each treatment level. Initially the rhizomes were freed from soil using pressurized air. Three treatments were applied:

- exposure to 65°C hot air (in an autoclave) for 30 minutes
- exposure to 121°C for 20 minutes
- exposure to room temperature (no heat treatment)

All samples were kept in pots where they had been covered with substrate (standard potting mix, see above) and left in an open greenhouse over winter without any thermic insulation and protection against loss of humidity until the rhizomes started to re-sprout in the next vegetative period. Additional to the treatments, three rhizomes, not subjected to heat treatment, were placed in pots without soil side-by-side with the other samples.

3 Results and discussion

3.1 Capacity of bacterial strains to promote plant growth

For the plant growth promotion assay, bacteria isolated from wheat seeds were used. The strains had shown in previous greenhouse experiments an improvement of germination and growth of wheat, a grass species. Therefore, the strains were tested on seed mixtures with a high percentage of grass seeds. In Figure 1 the results of fresh and dry green biomass are shown. When using the seed mixture B3, all bacterial strains tested caused a lower biomass production as compared to the control with significant reduction compared to the control (Figure 1 top). No significant effects were observed with B4 seeds. Both seed mixtures have almost the same amount of grasses (78% for B3 and 80% for B4), however, they have a different composition of Fabaceae species. B4 contains three different clover species, whereas B3 contains *Onobrychis*, clover and alfalfa. To find out, if certain plant species react differently to the strains, every single component of the mixture needs to be evaluated to find optimal seed mixtures for the specific strains.

Strain 4-3, which led to significant reduction of plant biomass production of B3 seeds is a *Pseudomonas* strain, which has already shown plant growth-promoting capacity on wheat under similar conditions. It is known that in some cases the outcome of plant-microbe interactions is highly plant genotype – dependent, which is obviously the case for strain 4-3. It is evident that a screening with many more strains needs to be performed to identify an optimal bacteria-seed combination resulting in plant growth promotion, especially at early plant growth stages.

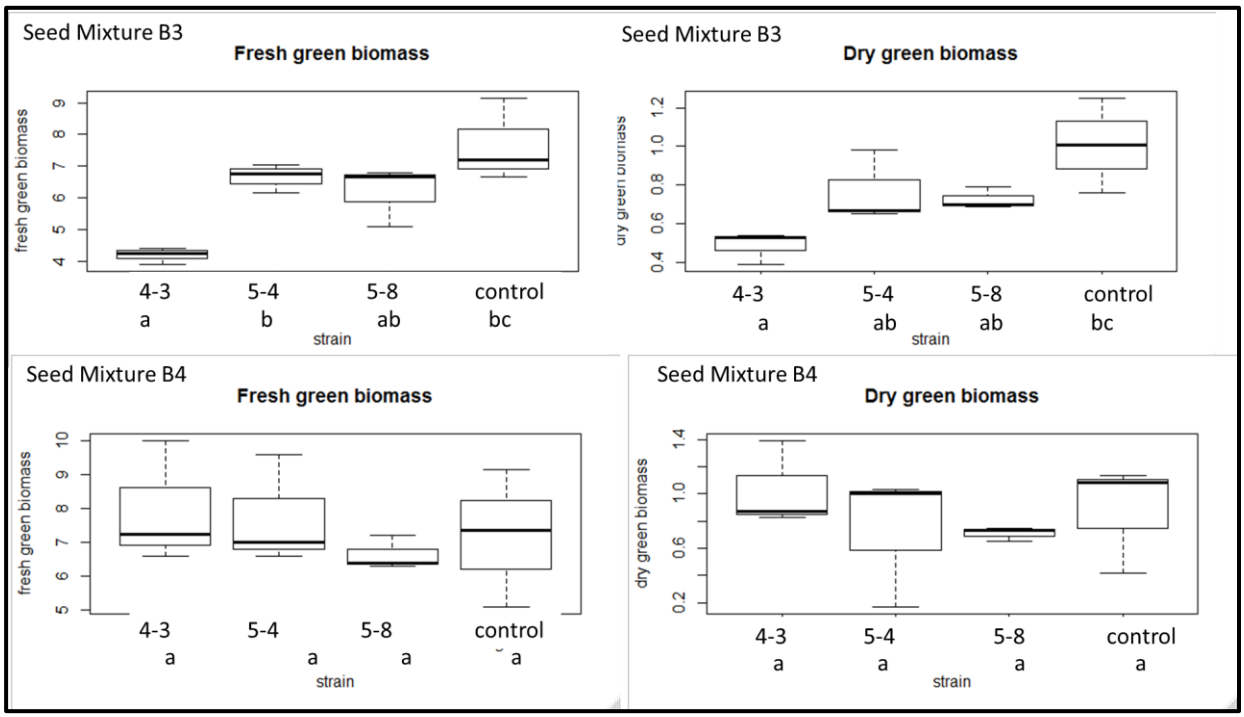


Figure 1: Fresh (left) and Green (right) b biomass obtained after inoculation of two seed mixtures (B3 and B4) with three different PGPB strains (4-3, 5-4 and 5-8). The letters indicate the significant differences between the treatments (P<0.05).

3.2 Inhibition of seed germination by ragweed-associated bacteria

For the two seed mixtures, B3 and B4, no reduction of germination of ragweed was observed (Figure 2). In the first experiment, conducted in March, the treatment with strain 6 (*Acidovarox* sp.) resulted in a lower germination rate in the B4 seed mixture compared to the non-inoculated control plants but the difference was not significant. In the second experiment, conducted in May, the strain had no effect on the germination of ragweed seeds. In the seed mixture B3 no inhibition was observed. In contrast, strain 473 (*Rhizobium*) increased the germination of ragweed significantly compared to the control in the B3 mixtures. Strain 444 (*Microbacterium*), which reduced the germination of ragweed in former experiments by 30%, did not show any effect on the germination of ragweed during these experiments. Strain 504 (*Bacillus*) was the only strain in the experiment, that (although not significantly) reduced the germination of ragweed in the seed mixture B4 compared to the control in the second experiments. The differences among both experiments may be explained by different temperature conditions in both experiments in the greenhouse (higher temperature in May). In the second experiment ragweed seeds germinated faster compared to March, and also more seedlings were obtained.

Overall, we observed variable results, still a common problem when applying biological agents. Different conditions cause different responses in the biological control agent and more rigorous screening is required to identify microorganisms showing more reproducible effects.

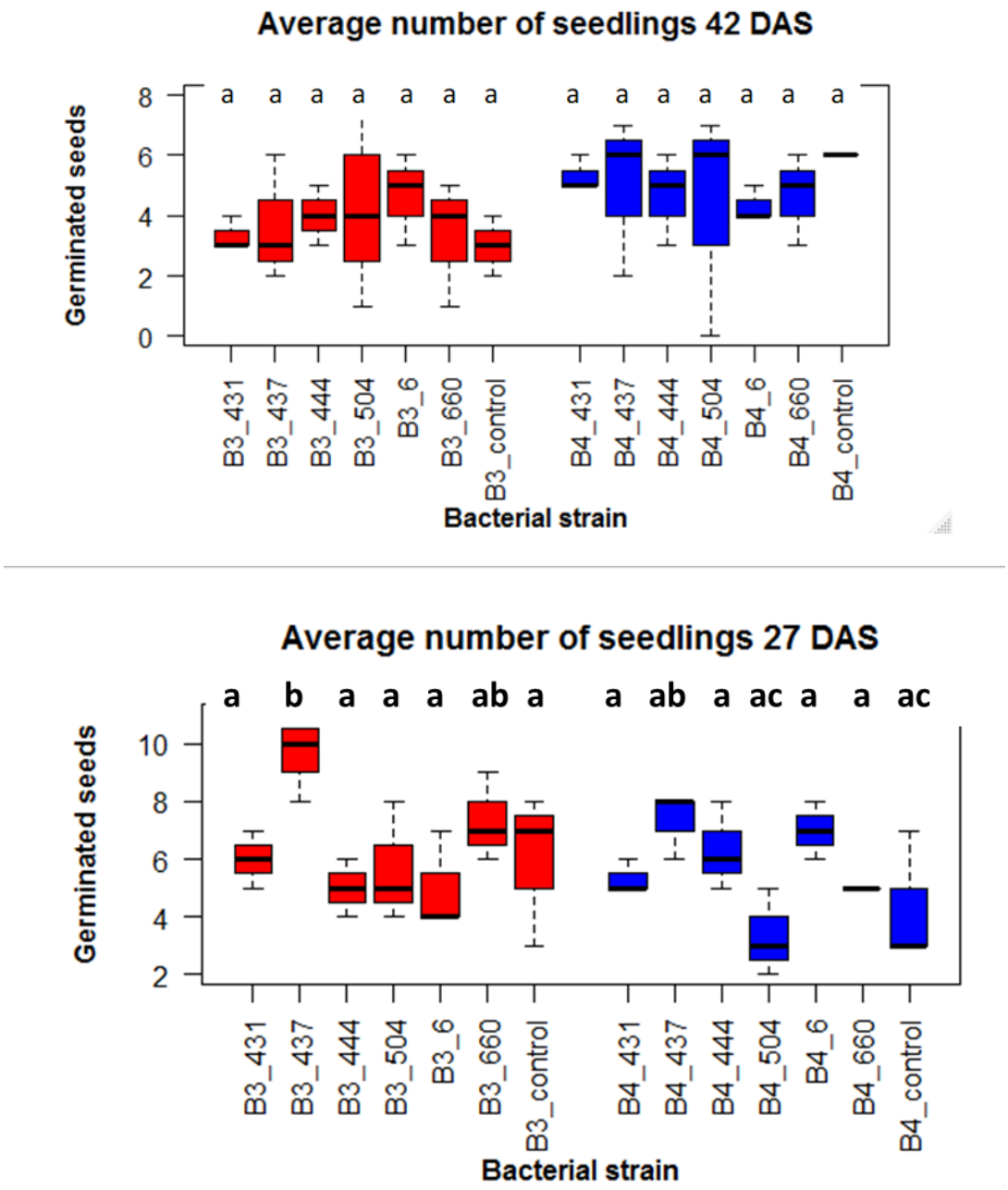


Figure 2: Germination of ragweed seeds grown together with two seed mixtures of endemic competitive grasses and clovers and treated with different PGPB strains. The top diagram is from the experiment conducted in March and the lower diagram is from the experiment in May. Two different seed mixtures (B3 and B4) and six bacterial strains (504, 444, 431, 660, 6 and 473) were used. Different letters indicate significant differences in each seed mixture ($P<0.05$).

It has been reported that *Festuca* spp. produce root exudates that outcompete many other plant species (Bertin et al. 2007). *Festuca rubra* produces meta-tyrosine which is known to act as a broad-spectrum phytotoxin on broadleaved plants. Both seed mixtures contain a high percentage of *Festuca* spp. In the seed mixture B3, almost 48% of the seeds are *Festuca* spp., of which 29% are *Festuca rubra*. In the seed mixture B4 only *Festuca rubra* is present in rather high amount (35%), which may cause the reduction of ragweed.

Based on the observed germination rate of ragweed seed grown together with both native seed mixtures, the seed mixture B4 resulted in better results in regard to ragweed inhibition. Our hypothesis is that high amount of *Festuca rubra* in the native seed mixture B4 together with strain 504 could bring about a more pronounced reduction of the germination of ragweed. However, more and larger tests are needed for confirmation.

The use of naturally occurring microorganisms for the biocontrol of IAPs is still in its infancy. Strains showing reproducible effects have to be identified and appropriate application practises developed. Also, safety as well as regulatory issues have to be addressed before application in the field.

3.3 Reduction of ragweed germination in the presence of Festuca

To prove the findings of 3.2 that high content of *Festuca rubra* in seed mixtures can reduce the germination, three different varieties were tested in the greenhouse together with ragweed seeds. Already after five days the ragweed seeds started to germinate in the different treatments. The variety Raisa germinated very quickly and after 12 days more than 50% of the seeds were germinated. At the same time point 40% and 20% of the varieties Smaragd and Melitta, respectively, germinated. After four weeks the trial was terminated and the results were analysed. Ragweed seeds showed in all three *Festuca rubra* treatments a lower level of germination (Figure 3) compared to the pots with only ragweed seeds. The highest reduction of germination was obtained in the treatment with the variety Raisa, which also showed best germination and this reduction was significant (P<0.03). This may be due to faster germination of this variety compared to the others. The variety Melitta showed the lowest germination rate (30%), followed by Smaragd with 53% and Raisa with 75%. The presence of well-established *Festuca* plants may prevent with their root exudates the germination of ragweed. The use of seed mixtures with a high percentage of *Festuca rubra* in greening mixtures for road verges may prevent the establishment of broadleaved IAPs like ragweed.

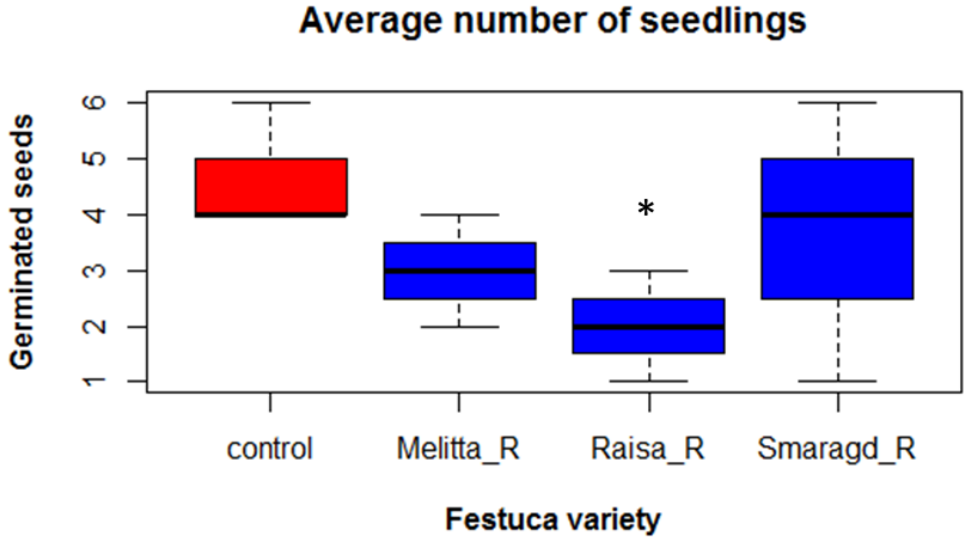


Figure 3: Ragweed germination in the presents of three different *Festuca rubra* varieties to inhibit the germination of ragweed in pots. Significant effects (P<0.05) are indicated with an asterisk.

3.4 *Development of biocontrol agents against* Impatiens glandulifera

3.4.1 Analyses of the genetic sequence of the bacterial strains isolated from I. glandulifera

In total 222 strains were isolated and characterized based on DNA sequence of the partial 16S rRNA gene. The largest group (25% of all strains) formed the members of the genus *Pseudomonas*. The second largest group were samples belonging to the genus *Microbacterium* (11%) and *Erwinia* strains occupied the third place (9%). *Pseudomonas* belongs to the class of Gammaprotobacteria which also represent with 109 strains the biggest class. To this class also the genera *Pantoea* (9 strains), *Stenotrophomonas* (14 strains) and *Erwinia* (20 strains) belong. The class of Actinobacteria with 51 strains is the second biggest class with *Microbacterium* (25 strains) and *Rhodococcus* (7 strains) as the most frequently found genera in this class. Twenty strains belonging to the class of Alphaprotobacteria were found with six strains belonging to *Rhizobium* and three strains to the *Sphingomonas*. Other classes found were 12 strains belonging to Bacillus, 11 strains to *Sphingobacteriia*, 10 strains to *Flavobacteriia* and 7 strains to *Betaproteobacteria*.

3.4.2 In vitro screening of Pseudomonas *and* Microbacterium *strains for germination inhibition*

In total, 48 *Pseudomonas* and 23 *Microbacterium* strains were tested for their ability to inhibit the germination of lettuce (*Lactuca sativa*) seed. The genera were selected because *Pseudomonas* was shown in in previous experiments to inhibit germination, whereas for *Microbacterium* strains have previously shown bioherbicidal activity (e.g. Cordovez et al. 2018; own un-published results). In this study, 13 strains significantly inhibited the germination, relative to non-inoculated control seed. Strain IG-R-15 (*Pseudomonas* sp.) brought about the greatest inhibition of seed germination (76% less germination), followed by strain IG-R-1.2 (*Pseudomonas* sp.) (65%). The other 11 strains showed much smaller effects ranging from 20 to 40% germination inhibition (Figure 4).

The 13 best-performing strains were tested again on lettuce seed germinating in soil without sterilization of their surface. As expected, the inhibitory effect on seed germination was smaller than in the sterile filter paper assay. Nonetheless, strains IG-R-1.2 and IG-R-15 again showed the greatest inhibitory effect (Figure 5).

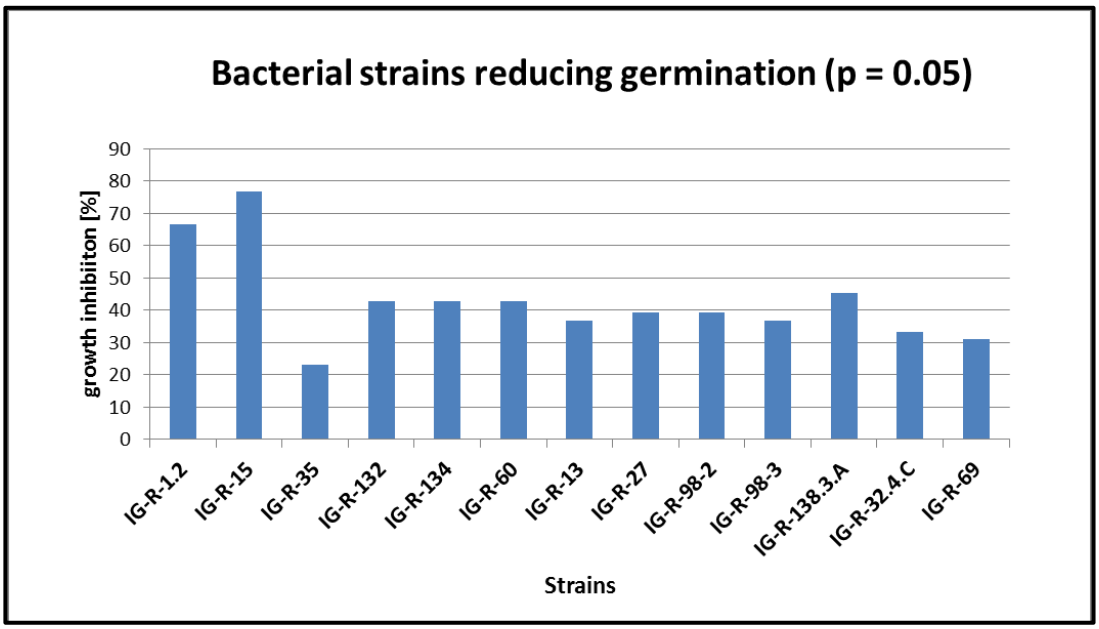


Figure 4: Results of significant germination inhibition in the in vitro assay of 13 strains (P<0.05). The results are in relation to the control treatment without bacteria. The control was set as 0% germination inhibition.

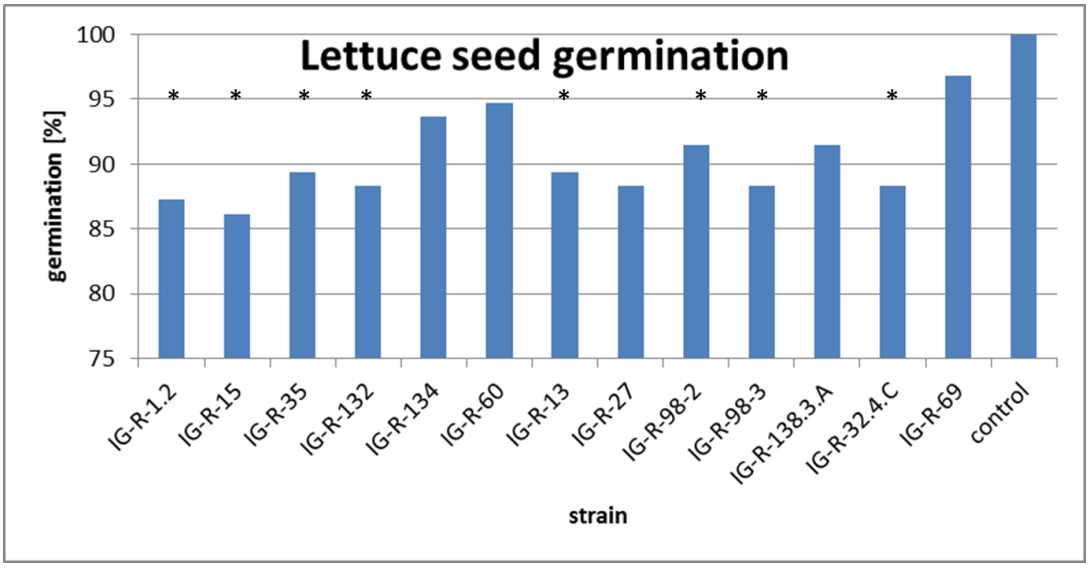


Figure 5: Results from the germination test in soil after inoculation of lettuce seeds with different bacterial strains. (Control; imbibed in bacteria-free growth medium). *; P<0.05.

The positive bacterial strains belong to both *Pseudomonas* and *Microbacterium*. The assignment to the different genus is summarized in Table 4.

Table 4: Assignment of the bacterial strains

Strain name	Genus
IG-R-1.2	*Pseudomonas* sp.
IG-R-15	*Pseudomonas* sp.
IG-R-35	*Pseudomonas* sp.
IG-R-132	*Pseudomonas* sp.
IG-R-134	*Pseudomonas* sp.
IG-R-60	*Pseudomonas* sp.
IG-R-13	*Microbacterium* sp.
IG-R-27	*Microbacterium* sp.
IG-R-98-2	*Microbacterium* sp.
IG-R-98-3	*Microbacterium* sp.
IG-R-138.3.A	*Pseudomonas* sp.
IG-R-32.4.C	*Microbacterium* sp.
IG-R-69	*Microbacterium* sp.

3.4.3 Dosage effects

To find out if a high dosage of bacterial cells can influence the growth of the seedlings, lettuce seeds were inoculated with different amounts of the corresponding bacterial suspensions. The isolates IG-R-98-2 and IG-R-98-2 did not show any significant influence on the root and shoot length in this assay when the seeds were inoculated with the bacteria (Figure 5). Both strains are assigned as highly related *Microbacterium* strains, showing the same 16S rRNA gene sequence but different appearance on plates.

Most of the strains have confirmed the results obtained from plate assays. Strains like IG-R-132, IG-R-134, IG-R-1.2 and IG-R-15 showed a reduction of root length formation depending on the dosage applied to the seeds (Figure 6). With higher concentrations, the root length was reduced. The effects on stem length were in most treatments not significant due to bacterial application (significance data not shown). The inoculation with strain IG-R-27 resulted in a significant root length reduction with the bacterial cell dosage of 10^7 cfu/ml.

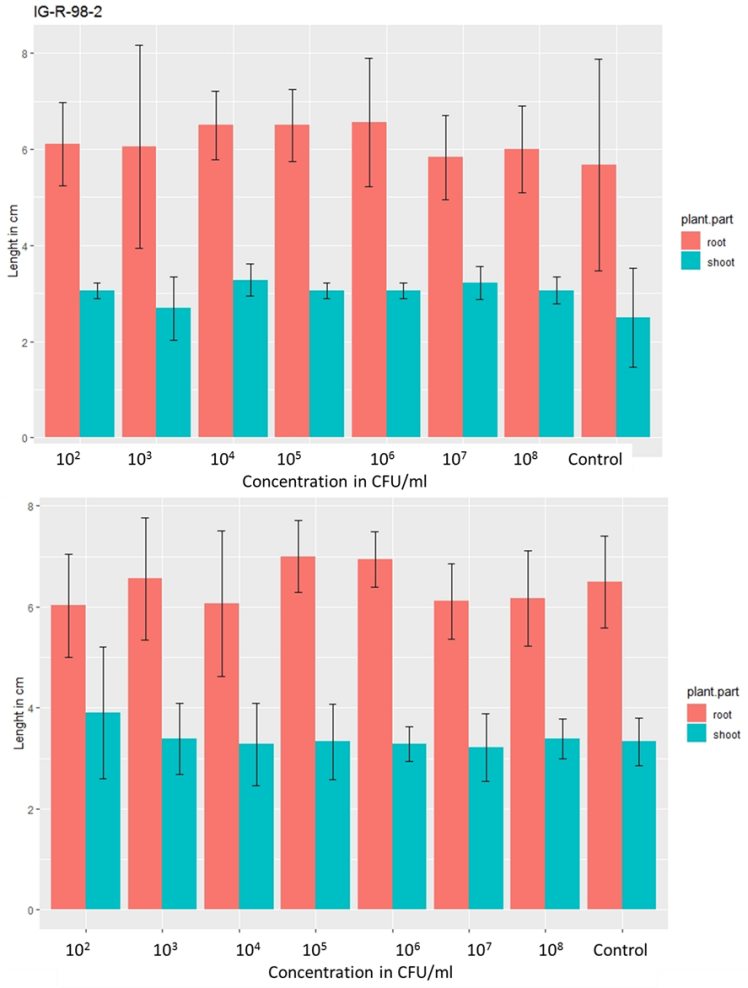
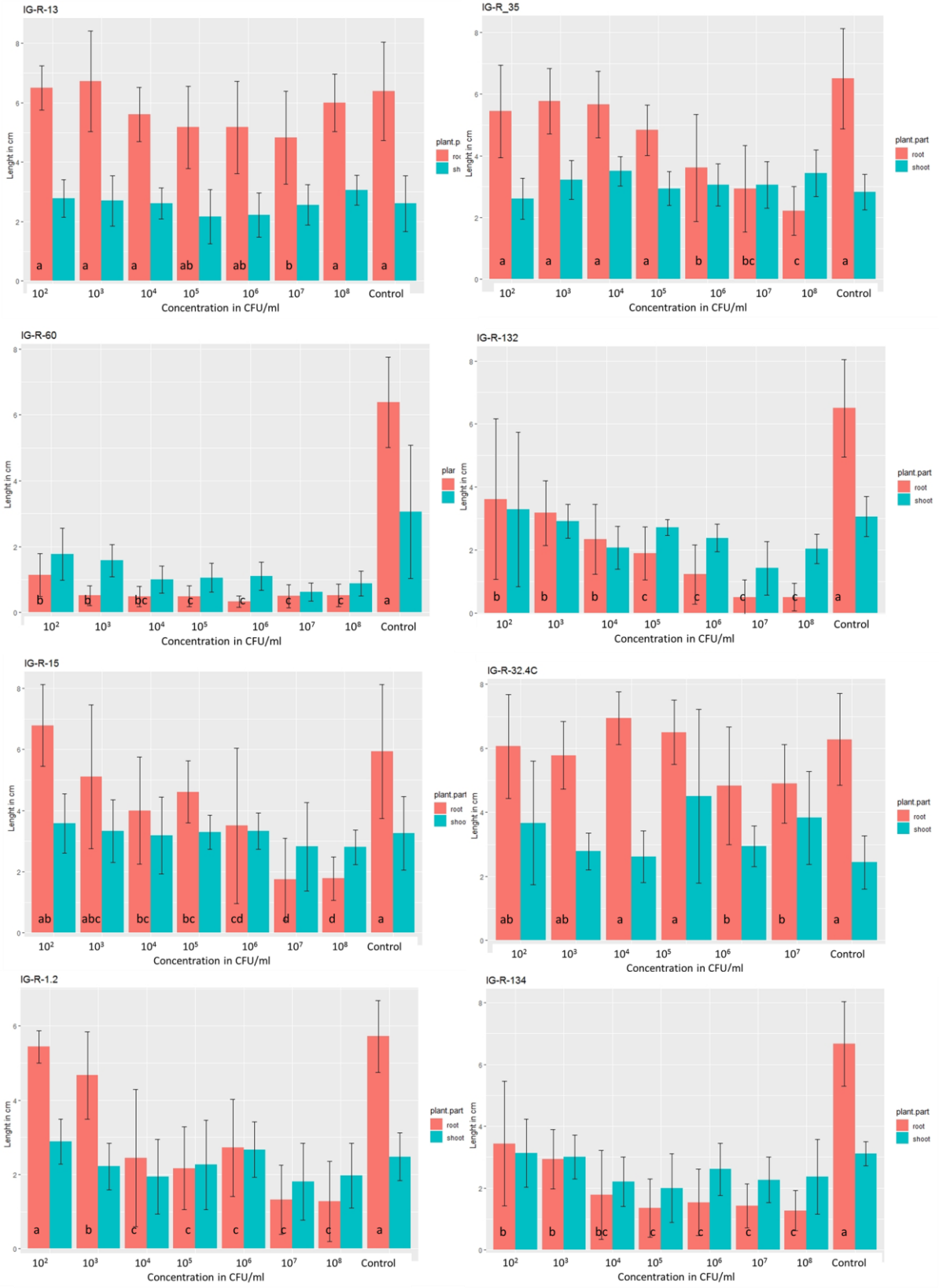


Figure 5: Dosage effects of bacterial inoculation on the root and stem growth of lettuce seedlings for the treatment with the strains IG-R-98-2 and IG-R-98-3. No significant differences were found ($P<0.05$).


IG-R-13
IG-R_35
IG-R-60
IG-R-132
IG-R-15
IG-R-32.4C
IG-R-1.2
IG-R-134
Lenght in cm
Concentration in CFU/ml
plant.part
root
shoot
Control

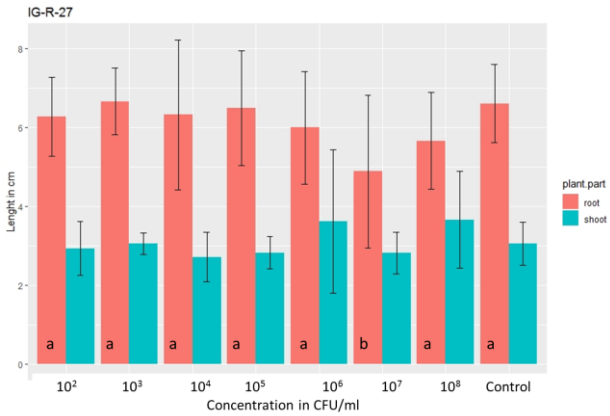


Figure 6: Root and shoot length of lettuce seeds inoculated with different bacteria in different bacterial concentrations. Letters indicate significant differences (P<0.05) for the root length. Shoot length values did not differ significantly.

In Figure 7 the size of the seedlings is shown. Inoculation with strain IG-R-69 applying a dosage of 10^5 cfu/ml had an effect on the seedling size. The seedling size of the seeds treated with the strain IG-R-138.3A decreased with an increase of the bacterial cell concentration.

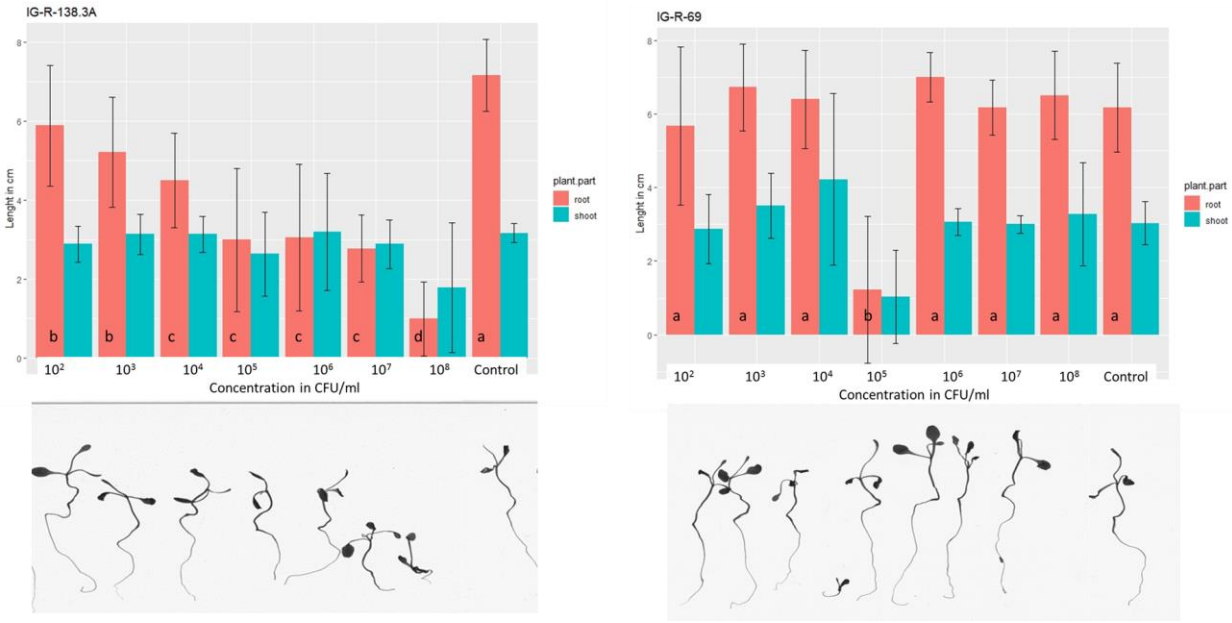


Figure 7: Effect of the bacterial dosage on the size of lettuce seedlings. Two selected bacterial strains were tested. Different letters indicate significant differences (P<0.05) for the root length. Shoot length values did not differ significantly. The pictures were taken with the root scanner and the software WinRHIZO (REGENT INSTRUMENTS).

3.4.4 In vitro indole acetic acid (IAA) production

All 13 bacterial isolates showing promising results in the lettuce assay were tested if they are able to produce indole acetic acid (IAA) *in vitro*. IAA belongs to the group auf auxins and at

low concentration the plant hormone stimulates plant growth but at high concentration it induces phytotoxicity (Grossmann 2010). The plant reacts to elevated auxin levels with inhibition of root and shoot growth, decreased internode elongation and leaf growth, and intensified green leaf pigmentation, accompanied by stomatal closure and an increase of reactive oxygen species (Grossmann 2010). The tested strains produced different amount of IAA *in vitro*. The strain IG-R-138.3A produced the highest amount of IAA in the presence of tryptophan as precursor (11 µg/ml) followed by strains IG-R32.4C and IG-R35 with 6.9 and 3.1 µg/ml, respectively. The strain IG-R-138.3A was the strain, which showed the inhibition of the root growth depending on the applied dosage (Figure 6), which could be due to the higher concentration of IAA at higher dosages. The strains IG-R32.4C and IG-R35 with lower amount of auxin production did not show any concentration-dependent growth inhibition.

3.4.5 *Greenhouse assays using seeds of* I. glandulifera *and the bacterial strain* Pseudomonas sp. *IG-R-60*

Strain IG-R-60 was tested in a greenhouse assay for inhibiting effects on the germination of *I. glandulifera*. In total 10 seeds in 10 replicates were sawn in pots and inoculated with IG-R-60 or left uninoculated. After one month only seven seeds germinated out of the 100 seeds in the IG-R-60 treatment and eight seeds in the control treatment without bacteria. Based on the low germination rate no conclusion could be drawn. The seedlings were measured for stem and leaf length and compared. Between the treatments no differences in the growth were observed although the stems were shorter in the IG-R-60 treated plants (Figure 7). However, because of the extremely low germination rate of the I*. glandulifera* seeds, no conclusion on the effect of the strain to the growth of *I. glandulifera* can be drawn. In the second treatment with IG-R-138.3A no seeds of *I. glandulifera* germinated at all.

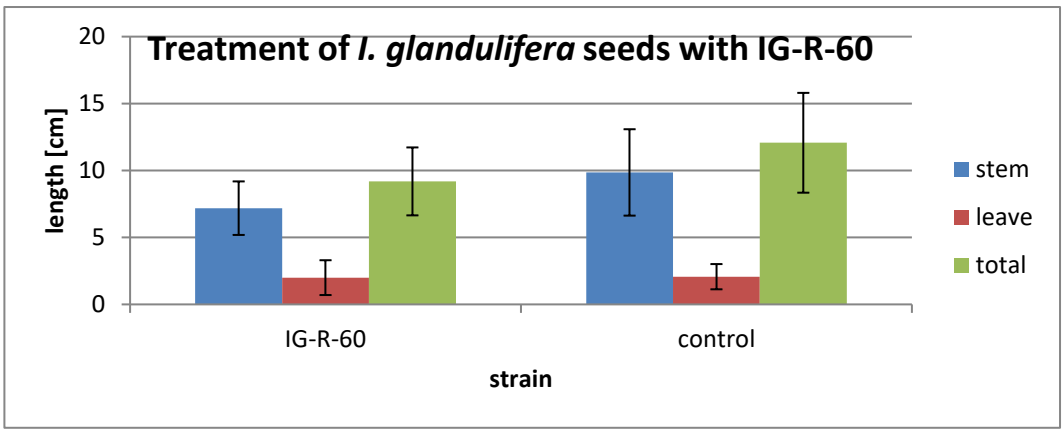


Figure 7: Length of seedling shoots and leave of *I. glandulifera* after the treatment with strain IG-R60.

3.4.6 *Non-target assay with seeds from* Impatiens walleriana *(Busy Lizzie)*

Busy Lizzie is an ornamental plant, which is one of the most relative to Himalayan balsam. To test for potential non-target effects, this plant species was used and different bacteria (as described in 2.3.3.) were applied in different concentrations. For every concentration three seeds in three replicates were treated. Unfortunately, only 50% of the seeds germinated independent from the bacterial application, i.e. also control seeds germinated very poorly. No significant differences of bacterial treatments and concentrations on shoot and root formation

were found. Due to the poor germination of seeds in general, the standard deviations were high. For strains IG-R-1.2 (belonging to the genus *Pseudomonas*) a significant decrease of plant growth was observed, but more tests are required to assess non-target effects of these strains.

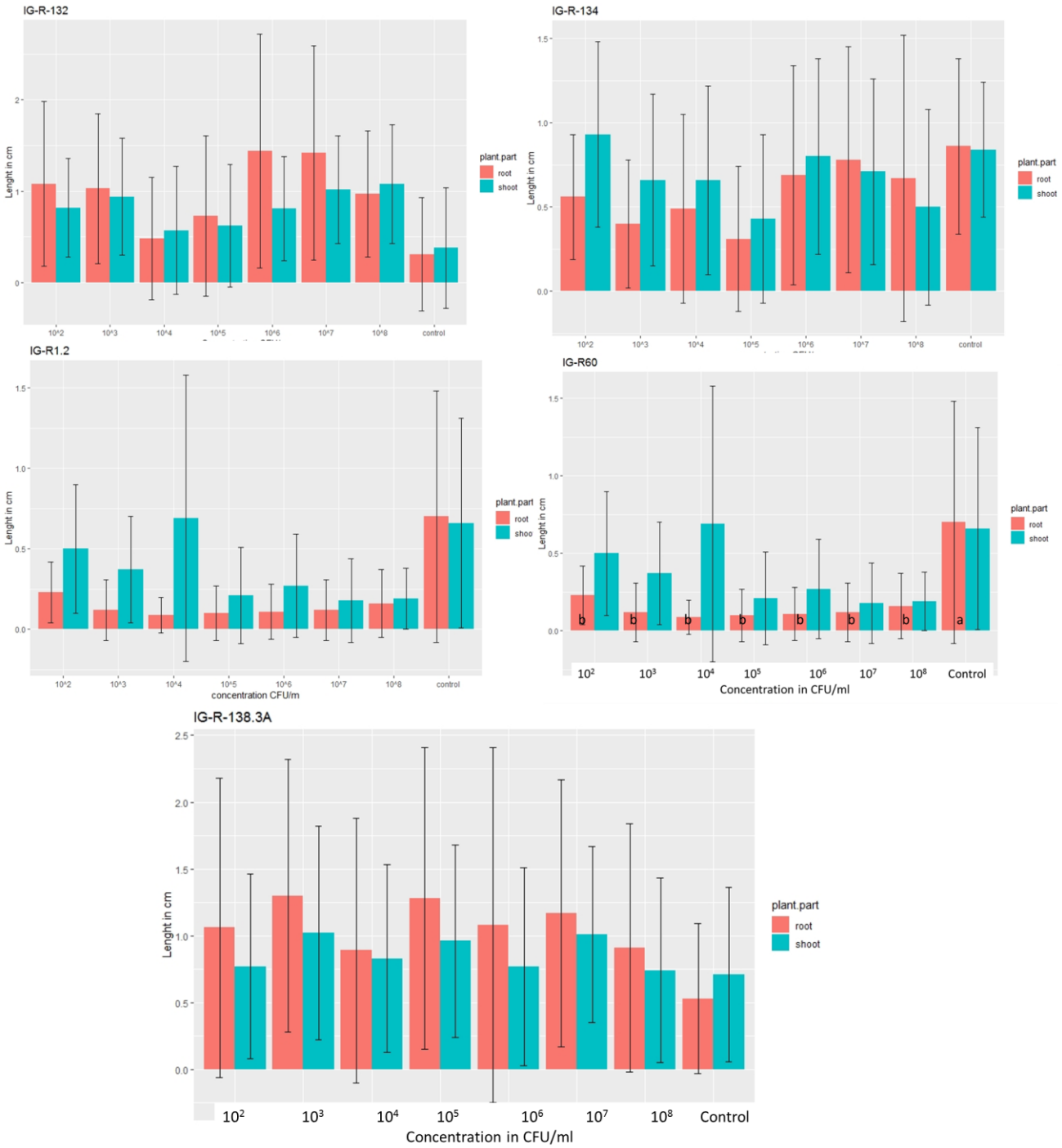


Figure 8: Results from the dose dependent assay with different bacterial solutions on Busy Lizzie. No significant effects were obtained (P<0.05).

3.4.7 *Results from the treatment of juvenile* Impatiens glandulifera

The strains (IG-R-138.3A and IG-R60 were applied to *I. glandulifera* plants, which were taken from an infested site. Before the treatment, they were kept for two weeks in the soil and watered regularly. The plants were cut above the first node and the bacteria were sprayed on the stems. The aim was to find out, if the applied bacteria can inhibit the regrowth of the plant after mowing. Based on the biology of the IAP we expected, that the plants would re-sprout from the nodes. Unfortunately, none of the cut plants started to regrow three weeks after cutting. Also, the control plants, which only received buffer treatment, did not produce new stems. Therefore, we cannot conclude on the effects of the applied bacterial strains.

3.5 *Experiments to inhibit the regrowth of* Fallopia japonica *rhizomes*

The aim of this initial trial was to find out if the *Fallopia japonica* rhizomes still sprout when 1) kept at normal cold temperature in winter without soil cover and 2) when treated with heat. The rhizomes were overwintered in a wirehouse in pots with or without soil. In early April first sprouts were visible on rhizomes that had received dry heat of 65°C for 30 min and on the untreated rhizomes (Figure 9 and 10). The untreated rhizomes sprouted fastest, but in the end, the 65°C treatment slowed the early regrowth, but did not significantly decrease regrowth of the rhizomes. In contrast, application of 121°C for 20 minutes completely killed the rhizomes; no rhizome developed sprouts. Surprisingly, those rhizomes that had been left over the winter without soil did not regrow either. They were apparently unable to develop new sprouts in spring (Figure 11). We will continue the observation to see whether the rhizomes re-sprout in the next vegetation period when they will be put back into soil.

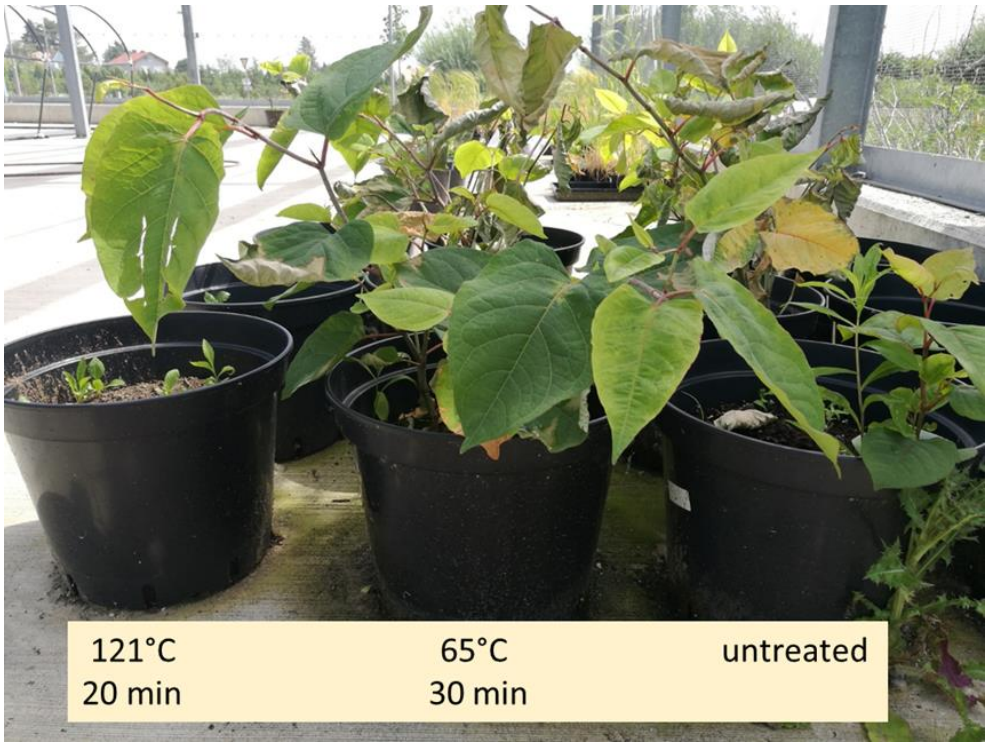


Figure 9: *Fallopia* rhizomes treated in autumn 2018 with different temperatures and times and the regrowth in spring 2019

Figure 10: Rhizomes of *Fallopia japonica* treated with different temperatures or left without soil. Left picture in April 2019, right picture June 2019

Figure 11: The rhizomes left overwinter without soil, no sprouts were visible

These results suggest that instead of energy-intensive IAP control methods, exposure to frost without soil, perhaps after ploughing, might be an alternative for the control of Japanese knotweed. Nonetheless, for effective removal of established stands of this invasive plant, mechanical separation of all rhizomes would be required and rhizomes should be exposed to frost for a longer period before they can be disposed of to avoid the risk of regrowth at the site of disposal. Consequently, none of the manipulations tried here fulfil the requirements of an efficient, low-cost, non-chemical control method of this robust plant.

4 Conclusions

4.1.1 Plant growth promotion of natural plant communities

PGPB are well known for their plant growth-promoting activities leading to better growth under stress conditions, better germination or better provision of nutrients. However, effects are best observed under stress conditions and the effects depend on a number of parameters such as compatibility between bacterial and plant genotype, nutrient or environmental conditions (e.g. Brader et al., 2017). One of the bottlenecks in the application of bacterial strains is also that they have to establish in the plant environment.

In this study the tested strains did not promote the growth of the plants of the seed mixtures B3 or B4. With seed mixture B3 one strain, strain 4-3 (*Pseudomonas* sp.), showed even growth reduction, although this effect was not seen with seed mixture B4. Interestingly, this strain showed growth promotion on two winter wheat varieties when tested in a greenhouse experiment. It is known that bacteria may differently interact with different genotypes or show different effects / phenotypes under different conditions (Brader et al., 2017). Obviously strain 4-3 has a narrow host range and reacts sensibly to different conditions and genotypes, whereas other strains, such as e.g. *Paraburkholderia phytofirmans* PsJN, have a broad host range (Mitter et al., 2013).

In agriculture PGPB are used to improve the crop productivity (Compant et al., 2019). PGPB assist in nutrient acquisition through phosphate solubilisation, siderophore production and nitrogen fixation as examples. Road verges are poor soils with low plant available nutrients and high salt and water stresses for the plant. The use of PGPB to promote the growth of competitive desired vegetation would be one way to reduce the growth of IAPs, but bacteria which have shown growth promotion in crop plants may not be transferred directly to seed mixtures used for greening. A screening campaign is needed with a large number of strains, ideally isolated from the target plants, and tested with different varieties of the target plant. Our results obtained clearly indicate that a rigorous selection of bacterial strains is required, which promote growth of many different plant species (found in natural populations) and do not exhibit inhibitory effects. Selected strains have to undergo rigorous field testing and require suitable formulations for seed coating. Once developed, seeds coated with such strains could favour the growth of desired populations and thereby limit the growth of invading plants.

4.1.2 Inhibition of ragweed seed germination by ragweed-associated bacteria

The use of bioherbicides against IAPs which only reduce the growth of the invasive plant would be a sustainable solution for IAP management. In previous studies at AIT, the strains tested in this study showed significantly reduced the germination of ragweed when tested in greenhouse experiments. Here, when ragweed and seed mixtures were used together, no effect was observed in regard to the reduction of ragweed seedlings due to bacterial inoculation. We observed an influence of the seed mixture on the germination of ragweed. Furthermore, the germination of ragweed was dependent on the temperature, when the experiment was conducted. Inoculation with the strain 437 (*Rhizobium* sp.) resulted in more seedlings of ragweed in the seed mixture B3, which was not observed in the first experiment seen in March with lower temperature in the greenhouse. The seed mixtures had almost the same composition of grasses and broadleaved plants, but in seed mixture B4 a higher amount of *Festuca rubra* is mixed in, which at least partly could explain the observed effects. The allelopathic effects of *Festuca rubra* was also tested (see below chapter 4.1.3).

We again observed a variation of bacterial phenotypes and probably the presence of other plants could have interfered with the inhibitory effects of the applied strains. We do not have the information on the colonization level of the applied strains and whether e.g. root exudates of neighbouring plants affect either the establishment or functionality of the applied strain. However, the tests included only six strains, which previously showed most promising results, and clearly, more comprehensive screenings are required including also accompanying vegetation of the IAP.

4.1.3 Test of different Festuca rubra *varieties on the reduction of germination of ragweed*

Based on the observations of previous experiments and literature reports we tested, whether *F. rubra* has an allelopathic effect on ragweed. Testing the three different *F. rubra* varieties clearly showed a reduction of the germination of ragweed, when both plant types were sown at the same time. The different varieties showed different results in regard to germination and inhibition of ragweed. In the experiment a difference between the varieties were found in the speed of the germination of the fescue and the reduction of ragweed. For future use the varieties should be carefully selected to have the greatest impact on the reduction of unwanted broadleaved plants. Bertin et al. (2017) tested 80 different fine fescue varieties and 8 showed a very strong weed suppression. The variety Raisa was in our experiment the variety with the strongest ragweed suppression.

Therefore, in the field test 2019 it was planned to seed Raisa as a competitor for ragweed. Unfortunately, due to the dry summer 2018, no new seeds of Raisa were available. Therefore, the three varieties from the harvest 2017 (Raisa, Smaragd and Melitta) were mixed together and seeded together with ragweed into the test site. The results will be analysed in the deliverable 3.3 for the field trials.

4.1.4 Impatiens glandulifera*-associated bacteria as bioherbicide*

For the control of *I. glandulifera* we started a new campaign and approach towards the development of a biocontrol solution. We successfully isolated bacterial strains, which we tested with lettuce as a frequently used indicator plant for phytotoxicity, as collected seeds from *I. glandulifera* did not germinate. We identified bacteria which significantly reduced the germination and growth of lettuce in small assays and with constant results. The most promising isolates serve as good basis for further experiments on *I. glandulifera* plants, which would be required to obtain information on the tested strains. After identification of strains showing the desired effects, dosage requirements, non-target effects and genotype dependency need to be assessed before starting large scale trials and the development of formulations and application procedures.

4.1.5 Storage of Fallopia japonica *after sterilization*

After the removal of the rhizomes from a contaminated area, the rhizomes have to be stored or destroyed. It is recommended to store the rhizomes in a depth of at least five meters on a

place with no soil movement. This is an expensive practice. In this study we performed experiments to find out at which temperature the rhizomes are prevented to re-sprout.

Our results from one vegetation period showed, that the 20 min sterilization at 121°C prevent the rhizomes to re-sprout, whereas sterilization at 65°C for 30 min was not effective. Our results showed, that methods like hot water, steam or hot foam will not reach the required conditions and therefore do not destroy the cells of the rhizomes completely. The methods are therefore a not suitable in the management of *F. japonica*.

The storage of the rhizomes without soil on concrete base plate may offer the possibility to avoid the need to bury rhizomes as we did not observe any sprouting under the conditions used in this project. We recommend to further evaluate for how long the rhizomes have to be stored before they can be composted. For small infestation sites or gardens, this would be a low-cost alternative.

4.1.6 Recommendation for road maintenance in regard to the control of IAPs

The application of microorganisms for plant growth promotion and control of IAPs is still in the research stage and further development is required. The unique potential to lower salt and drought stress makes them very useful to promote the wanted vegetation on road verges. For example, salt stress can be greatly reduced by applying bacteria showing ACC deaminase (1-aminocyclopropane-1-carboxylate) which lowers the ethylene concentration in the plant (Wang et al. 2016). Selection of bacteria from plants growing on the road verges using screening criteria for specific road conditions, should increase the chance to find potential bacteria with the ability to promote the road vegetation to outcompete IAPs. This approach is still in its infancy but may be a powerful solution in the future.

The use of specific fescue varieties in the greening of road verges is a cost-efficient way to outcompete broadleaved IAPs, which can be readily applied. During road construction seed mixtures containing a high percentage of fescue like the variety Raisa should be used for greening the verges. It may be worth to consider and further evaluate also other plant species showing allelopathic activities for greening road verges.

Long-term management of IAPs based on biologicals would be the most sustainable approach, although the research costs in the beginning are high. For example, the research and development (R&D) cost to control *Parthenium* weed with biologicals in Australia over 27 years reached Aus$ 11 Mio. However, the annual savings through improved pastures and reduced medical expenses are Aus$ 9 Mio, which already after two years recovered the investment in R&D (Page & Lacey 2006). The results show the great potential of biologicals also in regard to the control of IAPs on roads although the investment in R&D is high in the beginning.

5 Acknowledgements

The presented research presented in this report/paper/deliverable was carried out as part of the CEDR Transnational Road Research Programme, Call 2016. The funding for the research

was provided by the national road administrations of Austria, Germany, Ireland, Norway, Slovenia, Sweden and the Netherlands.

6 References

Adachi N. Terashima I, Takahashi M. (1996) Central die-back of monoclonal stands of *Reynoutria japonica* in an early stage of primary succession on Mount Fujii. *Annals of Botany* 77: 477-486

Ahemad M, Kibret M. (2014) Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *J King Saud University - Sci* 26: 1-20

Bale J, van Lenteren J, Bigler F. (2008) Biological control and sustainable food production. *Philos Trans R Soc Lond B Biol* Sci. 363: 761-76.

Becker E, Shamoun SF, Hintz WE. (2005) Efficacy and environmental fate of *Chondrostereum purpureum* used as a biological control for red alder (*Alnus rubra*). *Biol. Control* 33: 269-277

Bertin C, Weston LA, Huang T, Jande, G, Owens T, Meinwald J, Schroeder FC. (2007). Grass roots chemistry: meta-Tyrosine, an herbicidal nonprotein amino acid. *Proc Natl Acad Sci U S A* 104: 16964-16969.

Boyetchko SM. (1997) Efficacy of rhizobacteria as biological control agents of grassy weeds Proceedings of the Soils and Crop Workshop volume 97: 20-21.

Brader G. Compant S Vescio K, Mitter M, Trognitz F, Ma L, Sessitsch A. (2017). *Ecology and Genomic Insights into Plant-Pathogenic and Plant-Nonpathogenic Endophytes*; *Annu. Rev. Phytopathol*., 55: 61 - 83.

Compant S, Samad A, Faist H, Sessitsch A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* In press

Cordovez V, Schop S, Hordijk K, Dupré de Boulois, Coppens F, Hanssen I, Raaijmakers JM, Carrión V (2018) Priming of Plant Growth Promotion by Volatiles of Root-Associated *Microbacterium* spp. *Appl. Environ. Microbiol*. 84: e01865-18

De Jong MD. (2000). The BioChon story: deployment of *Chondrostereum purpureum* to suppress stump sprouting in hardwoods. *Mycologist* 14: 58-62.

Duke SO. (2007). The emergence of grass root chemical ecology. *Proc Natl Acad Sci U S A* 104: 16729.

Fuchs A, Pichler-Koban Ch, Elmenreich W, Jungmeier M. (2018) Game of clones: Students model the dispersal and fighting of Japanese Knotweed (*Fallopia japonica*). Book chapter in Invasive Species (Working Title) DOI: 10.5772/intechopen.82873

Grossmann K. (2010) Auxin herbicides: current status of mechanism and mode of action. Pest Manag Sci 66: 113-20.

Haas D, Defago G. (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol*. 3: 307-319.

Hayat R, Ali S, Amara U, Khalid R, Ahmed I. (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann Microbiol* 60 :579-598

Lugtenberg B, Kamilova F. (2009) Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol*. 63: 541-556.

Lygis V, Bakys R, Burokienė D, Vasiliauskaitė D. (2012) *Chondrostereum purpureum*-based Control of Stump Sprouting of Seven Hardwood Species in Lithuania. *Baltic Forestry* 18: 41-55.

Mantelin S, Touraine B. (2004) Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. *J. Exp. Bot*. 55: 27-34.

Mitter B, Petric A, Shin MW, Chain PSG, Hauberg-Lotte L, Reinhold-Hurek B, Nowak J, Sessitsch A. (2013) Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. *Front. Plant Sci.* 4: article 120

Mitter B, Pfaffenbichler N, Sessitsch A. (2016). Plant-microbe partnerships in 2020. *Microbial Biotechnol.* 9: 635– 640

Pfeiffer S, Mitter B, Oswald A, Schloter-Hai B, Schloter M, Declerck S, Sessitsch A. (2017) Rhizosphere microbiomes of potato cultivated in the High Andes show stable and dynamic core microbiomes with different responses to plant development. *FEMS Microbiol. Ecol*. 93 fiw242.

O'Callaghan M. (2016) Microbial inoculation of seed for improved crop performance: issues and opportunities. *Appl. Microbiol. Biotechnol*. 100: 5729-5746

Page AR, Lacey KL. (2006) Economic impact assessment of Australian weed biological control. CRC for Australian Weed Management. https://trove.nla.gov.au/version/46537981

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y. (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321: 341-361.

Sarwar M, Kremer RJ. (1992). Determination of bacterially derived auxins using a microplate method. *Lett. Appl. Microbiol*. 20: 282-285.

Tanner, R. 2017. Information on measures and related costs in relation to species included on the Union list: *Impatiens glandulifera*. Technical note prepared by IUCN for the European Commission.

Toumatia O, Compant S, Yekkour A, Goudjal Y, Sabaou N, Mathieu F, Sessitsch A, Zitouni A. (2016) Biocontrol and plant growth promoting properties of *Streptomyces mutabilis* strain IA1 isolated from a Saharan soil on wheat seedlings and visualization of its niches of colonization. South African Journal of Botany. 105: 234 - 239.

Trognitz F, Hackl E, Widhalm S, Sessitsch A. (2016) The role of plant–microbiome interactions in weed establishment and control. *FEMS Microbiology Ecology* 92: fiw138-fiw138.

Tworkoski TJ and Glenn, DM. (2012). Weed Suppression by Grasses for Orchard Floor Management, *Weed Technology* 26: 559-565.

Wang QY, Dodd IC, Belimov AA, Jiang F. (2016). Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase growth and photosynthesis of pea plants under salt stress by limiting Na^+ accumulation. *Funct. Plant Biol.* 43: 161–172.