ELSEVIER

Journal of Arid Environments 61 (2005) 227–247

Journal of Arid Environments

www.elsevier.com/locate/jnlabr/yjare

A survey of environmental and biological factors (*Azospirillum* spp, *Agrobacterium rhizogenes, Pseudomonas aurantiaca*) for their influence in rooting cuttings of *Prosopis alba* clones

P. Felker[a,c,*], D. Medina[b], C. Soulier[b], G. Velicce[c], M. Velarde[a], C. Gonzalez[b]

[a]*Universidad Catolica de Santiago del Estero, Santiago del Estero, Argentina*
[b]*Facultad de Agronomia y Agroindustrias, Universidad Nacional de Santiago del Estero, Santiago del Estero, Argentina*
[c]*INSIBIO, Universidad Nacional de Tucuman, Tucuman, Argentina*

Received 11 September 2003; received in revised form 16 August 2004; accepted 14 September 2004
Available online 2 December 2004

Abstract

To improve the rooting percentages of recently identified multi-purpose clones of *Prosopis alba* (algarrobo blanco), a survey was conducted of various plant growth promoting rhizobacteria and environmental factors. Four strains of *Agrobacterium rhizogenes* were examined as previous reports indicated this bacterium had stimulated rooting of difficult to root woody species, both by genetically transforming the tissue around the pericycle to induce roots, and without transforming the roots by secretion of growth promoting substances. *Azospirillum brasilense* was examined as this bacterium has been shown to stimulate growth in legumes by secretion of growth promoting substances, especially auxins. *Pseudomonas aurantiaca* was examined as a biocontrol agent for *Fusarium*, which we believe is an important pathogen in rooting *Prosopis*. The effect of the root temperature was examined by comparing mist benches with and without bottom heat. The influence of age of the stock plant on the rooting was examined by comparing the rooting of 21-1 year old plants vs. 4 clones from 4-year-old stock plants. Great variability was encountered in the experiments, which precluded definitive conclusions. Nevertheless, the most important effect in stimulating rooting was

*Corresponding author. D'Arrigo Bros, P.O. Box 850, Salinas, CA 93902, USA. Tel.: +1 831 235 2847.
E-mail address: peter_felker@hotmail.com (P. Felker).

doi:10.1016/j.jaridenv.2004.09.010

obtained by increasing the root temperature of the cuttings to about 33 °C, even when daily maximum air temperatures were 40 °C. The second most important factor seemed to be the age of the stock plant. Future research should be aimed at methods to rejuvenate the tissue, possibly by grafting onto immature seedlings. Of the rhizobacteria, the *Pseudomonas* seemed to have the greatest potential. *Azospirillum*, which often serves as "a helper bacterium"may have stimulated rooting in a synergistic way that is difficult to measure. The Tiger 232 strain of *A. rhizogenes* was selected from among the 4 *A. rhizogenes* strains as giving some of the most consistent results. When indolebutyric acid, napthaleneacetic acid, thiamine HCl, 33 °C root temperatures were combined with *A. rhizogenes* and *Azospirillum*, the mean rooting percentage and number of roots per cutting for 21-year old *P. alba* plants were 80% and 30, respectively. In contrast, 50% rooting was observed for four, 4-year-old stock plants. The rooting parameters for the young stock plants are much greater than obtained in earlier work on *Prosopis* (80% rooting with a maximum of 12 roots/cutting) and suggests that in spite of lack of significant treatment effects, these rhizobacteria may stimulate rooting.

Keywords: Agroforestry; Plant growth promoting rhizobacter; Nitrogen fixation

1. Introduction

Arboreal species of the nitrogen fixing genus *Prosopis* are important in tropical, subtropical and semi-temperate arid regions for fuelwood, as a source of high-quality furniture wood, soil enrichment/rehabilitation and for their high sugar content pods useful for human and animal food (Pasiecznik et al., 2001; Felker, 2005). The useful arboreal *Prosopis* are commonly known as mesquite in India, Pakistan, Yemen and Sahelian Africa, Bayahonde in Haiti, Cuyui in Venezuela, Kiawe in Hawaii and algarrobo in Peru and Argentina (Pasiecznik et al., 2001).

Multi-purpose *Prosopis* clones have recently been reported that have been selected for rapid growth, high production of pods, pods that are highly palatable for human food uses (Alban et al., 2002; Felker et al., 2001) and that have grown in seawater salinities (Velarde et al., 2003). A recent economic analysis of various management/genetic improvement scenarios suggested that under the lowest rainfall regimes, use of fast-growing clones would change the internal rate of return of plantations from 10% to 19% (Felker and Guevara, 2003).

Unfortunately routine uniformly high rooting of *Prosopis* cuttings has not been achieved except for the Peruvian *Prosopis pallida* clones which are easier to root than the other species (Alban, 2003, pers. comm.). This is in spite of more than 20 years of research involving use of various auxins and auxin conjugates (Felker and Clark, 1981), growth chamber trials evaluating environmental parameters (Klass et al., 1984), analyses of fungicides (Klass et al., 1986), rooting media (Wojtusik et al., 1994) and fertilization regimes (DeSouza and Felker, 1986). While the non-mist propagation system that uses translucent, high-humidity boxes with subirrigation have proved highly successful for rooting tropical *Prosopis juliflora* (Leakey et al., 1990) we have had very poor success with this system on the *Prosopis alba* which is much more difficult to root.

The asexual propagation of woody leguminous plants in general and *Prosopis* in particular is difficult and erratic due to factors such as light intensity, photoperiod, temperature, juvenile vs. adult physiological stages, exogenous hormone additions and disease. These interacting factors make it difficult to divide the research on rooting of cuttings into discreet tasks that can be solved in linear fashion and necessitates a broad approach in which the main factors are investigated at the same time. Owing to high variability, treatments are seldom significant. Our approach has been to select the treatment with the greatest rooting from the current experiment, to be used in the next experiment. The rationale for this approach is we assume that the variation will be random and that with enough cycles this variability will cancel out to permit development of a successful rooting protocol.

In addition to use of manipulation of the environmental conditions of the stock plant and cuttings and exogenously applied hormones to stimulate rooting, "Plant Growth Promoting Rhizobacteria (PGPR)" have recently been used to stimulate root growth. *Azospirillum* spp have found to have stimulative effects on the dry weight growth of leguminous plants even when the appropriate rhizobia were present, evidently by secretion of plant growth promoting substances (Burdman et al., 1997). Various *Pseudomonas* species have shown to be effective in controlling pathogenic fungi, especially *Fusarium* (Paulitz and Belanger, 2001; Pal et al., 2001; Rosas et al., 2001) which we have found to be a problem in rooting *Prosopis* cuttings.

Agrobacterium rhizogenes, known for hairy root formation in carrot disks and apple trees may induce adventitious rooting in woody plants by incorporating the *rolB* gene and/or by secretion of compounds that stimulate rooting (Haggman and Aronen, 2000).

While *A. rhizogenes* is technically a pathogenic bacterium to plants, no dramatic crop loss, nor pathological symptoms exist in plants transformed by *A. rhizogenes* (Strobel and Nachimas, 1985). Unlike *A. tumefaciens* which transforms most dicotyledonous plant tissues into tumors, *A. rhizogenes* apparently restricts itself to the pericycle … the organism does not destroy or otherwise degrade tissue and seems to be compatible with plant cells in culture (Strobel and Nachimas, 1985). *A rhizogenes* does not produce spores that would compound the elimination of this bacterium once the desired stimulation of rooting had taken place (Haggman and Aronen, 2000). *A. rhizogenes* treated bare root olive trees maintained an impressive and significant lead in growth and flowering vs. untreated controls during the first 4 years after planting in Israel (Strobel and Nachimas, 1985). Since *Prosopis* has an optimum rooting temperature about 35 °C while *A. rhizogenes* has an optimum temperature for infection of about 23 °C with no infection at 37 °C, (Whiteman et al., 1988) this discrepancy in optimum temperatures is a disadvantage for rooting *Prosopis*.

The first report of *A. rhizogenes* having a beneficial effect on *Prosopis* was by Caro et al. (2003) who reported that it increased rooting of *P. chilensis* in tissue culture systems. There are considerable reports of the benefits of *A. rhizogenes* inducing roots in tissue culture systems (see review by Haggman and Aronen, 2000). We are aware of no commercial forest plantings derived from tissue culture due to the high cost of tissue-cultured plants, but there are many clonal plantations produced from

rooted cuttings. Thus, we felt it important to examine the use of *A. rhizogenes* in a greenhouse environment, taking special precautions to avoid its spread as a pathogen. In addition to the early work of Strobel and Nachimas (1985) that found beneficial effects of *A. rhizogenes* in a greenhouse environment, a few other research groups have reported benefits of this bacterium on woody species in a greenhouse environment i.e. Aronen et al. (1996) for Scots Pine and Hatta et al. (1996) on jujube softwood cuttings. If the objections to use of genetically transformed plants could be legitimately resolved, possibly the ultimate solution to the rooting of difficult species would be the incorporation of the rooting gene *rolB* from *A. rhizogenes* into the desired plant as demonstrated by Zhu et al. (2001) for apples. These workers found 83–100% rooting for transformed plants vs. less than 1% for control plants. Alternatively, the work of McAfee et al. (1993) and Aronen et al. (1996) who found that *A. rhizogenes* significantly stimulated rooting without genetically transforming the plants, would alleviate the concerns over release of genetically modified organisms with the benefits of increased rooting.

2. Materials and methods

The rooting of cutting experiments occurred in the summers of (2001/2002 and 2002/2003) in a greenhouse of the Universidad Nacional de Santiago del Estero, Santiago del Estero, Argentina at 27°S latitude. No environmental controls were possible other than opening and closing the greenhouse sidewalls.

A system whose mist frequency was controlled by a combination of the solar intensity and the temperature, was used to adjust the amount of water on the unrooted cuttings in proportion to the evaporative demand (Felker submitted). In summary, the controller for this light/temperature mist system (Solar 3B-12 V DC model with thermostat option) from Davis Engineering (www.davisengineering.com) was powered by a 12 V truck battery permanently kept charged with a voltage regulator. The 12 V DC output from the Solar 3B controller operated a 12 V DC high pressure (4.2 MPa) pump (via a relay) that took its water source from an 800 l ground level water tank with a float valve to maintain maximum capacity. High-pressure mist nozzles (High Pressure Misty Mist from Dramm) were located 1 m above the bench and provided a low-volume fine mist. This system was capable of operating for more than 6 h when breaks in both the water and electrical supplies occurred.

The material used to provide the cuttings came from drip-irrigated stock plants that were established by grafting the 12 elite clones described in Felker et al. (2001) onto common rootstock. These stock plants were grown in 15 l containers in sandy soil and in the summer drip irrigated at 0800, 1300 and 1800 h until water was observed draining from the pots. In the first year, they were fertilized every two weeks by adding 2 ml of solid urea on top of the pots next to the dripper and by applying approximately 250 ml of a mixture of Peters 20-20-20 soluble fertilizer that contained the nutrients at the following final concentrations ($mg\,l^{-1}$); N—2, P_2O_5—2, K_2O—2, Mg—0.05, Ca—0.0, B—0.002, Cu—0.005, Fe—0.01,

Mn—0.005, Mo—0.00005, and Zn—0.005. In the last 2 years a fertilizer injector (Dosimatic) was used to constantly supply the stock plants with nutrients as well as the soil systemic carbofuran (Furadan) to control some insects. Owing to the harvest of all new 60 cm long shoots, every 4–6 weeks, the soluble nutrient mix above was fortified with additional N in the form of urea, Fe in the form of iron Sequestrene 138 (6% iron) and $MgSO_4$. Thus, in addition to the final concentrations used above, the drip irrigation solution also contained: urea-50 mg l^{-1}, iron Sequestrene 138—1 mg l^{-1}, $MgSO_4$—10 mg l^{-1}, and carbofuran (40% active)—2 mg l^{-1}

In Argentina due to co-evolution of *P. alba* with insects, a great variety of sucking and chewing insects occur on *P. alba* in the greenhouse that cannot be controlled by carbofuran alone. Thus, at weekly intervals the insecticide Cypermethrin was used at a final concentration of 200 mg l^{-1} as found effective to eliminate gall formation and chewing insects not controlled by the carbofuran (M. Ewens, Forestry Experimental Station Fernandez 2003, pers. comm.)

In the summer, approximately every 4 weeks, new shoots approximately 60 cm long could be harvested from these stock plants for cuttings. Three-node cuttings were used with all the leaves on the above-ground portion of the cuttings left remaining. The most tender material (less than 2 mm in diameter) and stems with brown coloration (greater than about 8 mm in diameter) were discarded using only stems with 100% green coloration. As previous experience indicated cuttings are sensitive to "overcrowding" each cutting was placed in an 8 cm diameter × 5 cm deep—250 ml circular plastic cup (normally used for ice cream) after drainage holes were made. Medium-size vermiculite was used as the rooting media. New vermiculite was used for each rooting experiment. The plastic pots were reused after being washed and then soaked in a sodium hypochlorite solution.

In the first 2 years, all of the treatments were conducted on a mist bench with bottom heat. In the last season, an additional mist bench was constructed without bottom heat to examine root temperature effects on rooting. The bottom heat was achieved by circulating hot water in a serpentine of copper tubing embedded in a low-weight charcoal matrix. The benches were covered with plastic sheeting. After each experiment, the sheeting was removed and brushed with a sodium hypochlorite system to eliminate fungal pathogens and any residual beneficial micro-organisms (*Pseudomonas*, *Azospirillum*, etc.) that might skew the rooting results.

In this work we have used the potassium salts of the auxin hormones, due to their greater water solubility that avoided use of organic solvents such as ethanol or dimethylsulfoxide (DMSO) as described earlier (Felker and Clark, 1981). To avoid problems with hormone instability, a small quantity of the hormone mixture (30 ml) was prepared less than an hour before use and discarded after the experiment. When used in a 50 ml beaker, this quantity of hormone provided sufficient liquid to dip about 400 cuttings 2 cm deep. The hormone concentrations were 6000 mg l^{-1} potassium salt of indolebutyric acid, 9000 mg l^{-1} of the potassium salt of napthaleneacetic acid and 800 mg l^{-1} thiamine HCl (Sigma Chemical Company).

All of the *A. rhizogenes* strains were non-engineered wild-type strains. The source for the bacteria used in these experiments was as follows: *Pseudomonas aurantiaca*,

Susana Rosas Universidad Nacional de Rio Cuarto, Argentina; *Azospirillum brasilense* BR 11005 used in the summer 2001/2002 from Universidad Nacional de Mar del Plata in Balcarce, Argentina, *Azospirillum* spp used in 2002/2003 from C. Bellone, Universidad Nacional de Tucuman where it was isolated from the wheat variety "Gigante Tranquena" growing in the locality of Las Talitas, Tucuman and was used to stimulate rooting of other woody species; *A. rhizogenes* strain coded "A" was strain ATCC 31798 from Dr. Caula Beyl, Plant Science Center, Alabama A&M University, Normal, Alabama, strain coded "B" was 232 "Tiger" from Dr. Gary Strobel, Dept. Plant Pathology, Montana State University, Bozeman, Montana, strain coded "C" was ATCC 31799 also from Dr. Caula Beyl, and strain "D" was LBA 9402 from Dr. Nestor Curvetto (Universidad Nacional del Sur, Bahia Blanca, Argentina). ATCC 31798 and ATCC 31799 are agropine strains derived from A4 wild-type strain and 232 and LBA 9402 are mannopine/agropine strains.

The bacterial inoculants were used in various ways. The *P. aurantiaca* after arrival by overnight courier at a concentration of 3.13×10^9 CFU g^{-1} in peat, was diluted ten-fold moist finely ground peat. The 2 cm distal portion of the cuttings was rolled in this peat mixture after the 4 s hormone dip. The *Azospirillum* inoculant was prepared by centrifuging a log phase growing culture (yeast extract-phosphate buffer with micronutrients) and resuspending in a physiological buffer to a concentration of 1×10^{11} bacteria ml^{-1}. The hormones were added directly to the *A. rhizogenes* liquid culture media. Unless otherwise specified, the cuttings dipped about 1.5 cm deep for 3 s. For the combination *Azospirillum*/*A. rhizogenes* treatment, 15 ml of each of the bacterial culture were mixed, the hormones added, and the cuttings dipped for about 3 s.

On one occasion we tested the hypothesis that it was necessary for longer contact with the bacterium. This was done as follows: (a) the cuttings were dipped for 3 s in the hormone mixture in water, (b) stuck in the vermiculite in the pots on the mist bench, and (c) 0.5 ml of a bacterial suspension containing 10^9 bacteria ml^{-1} (either *Agrobacteria*, *Azospirillum* or both) was pipetted onto the stem of cutting at the interface with the vermiculite rooting media.

To avoid confounding effects related to variability in mist distribution, light intensity etc, the cuttings were arranged in 8–10 blocks on the mist bench. Single replicates were used consisting of one pot per clone per treatment. We calculated the 95% confidence intervals of the number of roots per cutting or the length of the longest root per cutting based on the 8–10 replicates. It was not possible to calculate confidence intervals on the rooting percentages as this was the percentage of the 8–10 blocks.

About 4 weeks after initiation of the experiment, the root system was gently removed from the pots, the vermiculite gently shaken to remove the bulk of the vermiculite, the cuttings dipped in water containing 1% sodium hypochlorite as a disinfectant, then a 1000 mg^{-1} solution of oxytetracycline and the number of roots per cutting (zero = no rooting) and the length of the longest root measured.

3. Precautions to prevent spread of *A. rhizogenes* from the greenhouse

In most tissue culture experiments where *A. rhizogenes* is used to induce rooting, the antibiotic cefotaxime is added (at 500 mg l^{-1}) to kill the bacteria when it is assumed that the *Agrobacterium* has had its effect on rooting the cuttings. As this antibiotic is prohibitively expensive for greenhouse work, we examined the sensitivity of theses 4 *A. rhizogenes* strains to the common, inexpensive agricultural antibiotic oxytetracycline and found that the most resistant *A. rhizogenes* strain was killed at a concentration of about 35 mg l^{-1}. Moore et al. (1979) also reported that *A. rhizogenes* was killed by the similar antibiotic tetracycline at a concentration of 20 mg l^{-1}.

For the initial experiments using *A. rhizogenes*, all persons entering and leaving the area of the greenhouse were required to wear rubber boots and to step in a sodium hypochlorite solution before and after entering. One week after initiation of the experiment and every week thereafter, a 2% solution of sodium hypochlorite was sprayed below the mist bench and surrounding areas. One week prior to the end of the experiments, oxytetracycline was introduced into the mist system water (through the 800 l storage tanks) at a concentration of 50 mg l^{-1}. During the cutting evaluation, the cuttings were dipped in 1000 mg l^{-1} oxytetracycline. At the end of the experiment, all of the soil and pot media was sealed in garbage bags and treated with sodium hypochlorite for 3 days. After several experiments in which the marginal effect of *A. rhizogenes* on rooting was noted, and after realizing that summer temperatures in this location of 35–42 °C were far outside the range for *A. rhizogenes*, these procedures were reduced to only (1) adding oxytetracycline to the mist system prior to evaluation, (2) dipping the cuttings in both sodium hypochlorite and oxytetracycline and (3) disposal of the rooting media after treatment with sodium hypochlorite.

4. Results

A comparison of the effects of *A. brasilense* BR 11005 and *P. aurantiaca* on 6 clones of *P. alba* at two dates in the summer of 2001/2002 is shown in Fig. 1. All of the treatments had the basic hormone treatment of 6000 mg l^{-1} IBA, 9000 NAA mg l^{-1} and 800 mg l^{-1} thiamine HCl. At both the first and second evaluation date inclusion of *P. aurantiaca* improved the rooting percentages of clones B7F6A4, B1F52A3 and B2F4A3 but not for the other clones. On the first evaluation date, clone B2F17A2 had yellowish foliage while on the second evaluation date the foliage was dark green and in good condition, which probably was responsible for the greater rooting at the second evaluation date. This clone is generally quite easy to root but very few of the rooted cuttings survive due to extreme susceptibility to *Fusarium* in the recently rooted cutting stage (Mature stock plants seem to have no problem with regard to *Fusarium* pathogens). There was no consistent trend in the rooting percentage of the cuttings as a result of *Azospirillum* inoculation within or

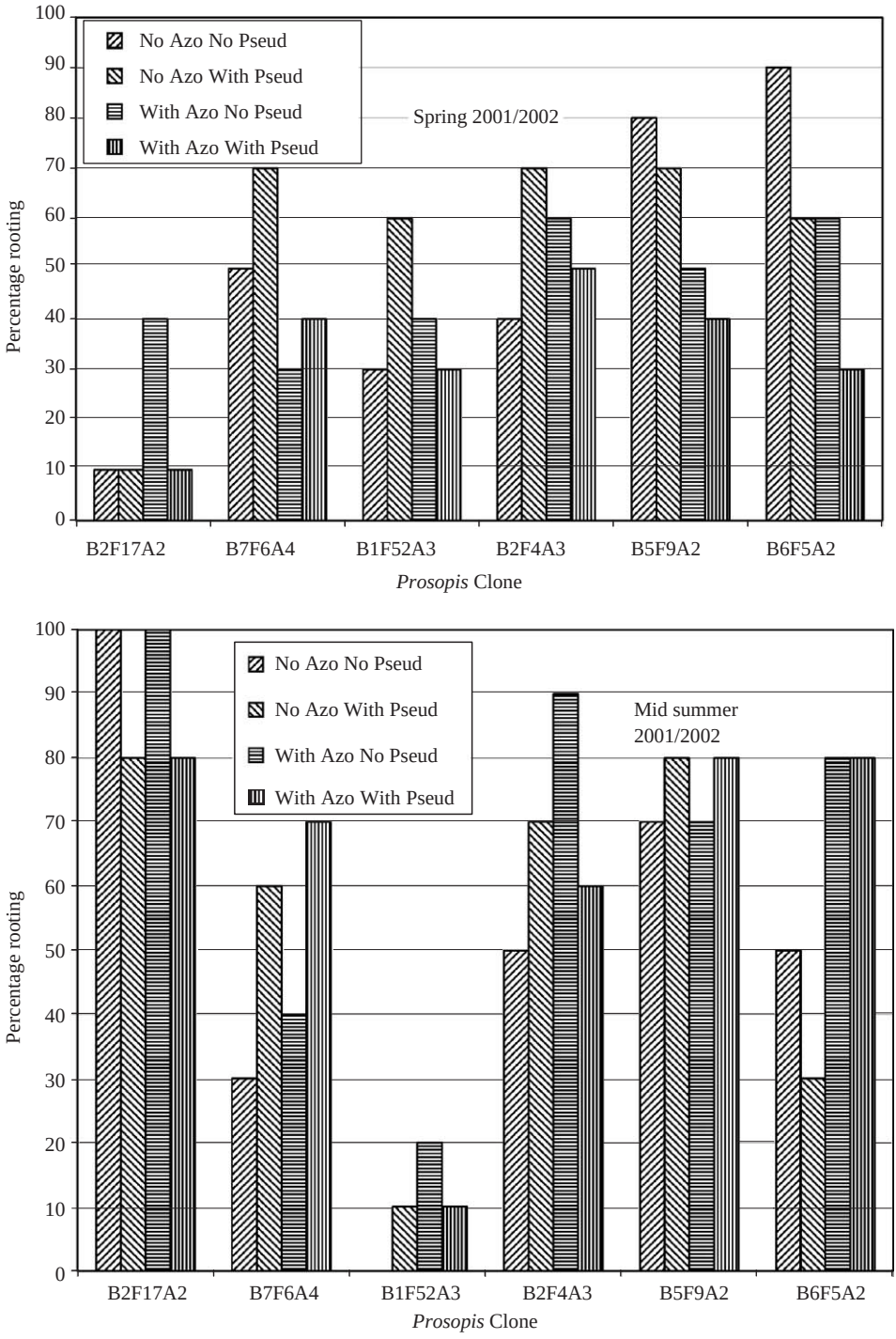


Fig. 1. Influence of *A. brasilense* and *P. aurantiaca* on the rooting percentage of *Prosopis* clones when inoculated with a 3 s dip containing the hormones described in the methods.

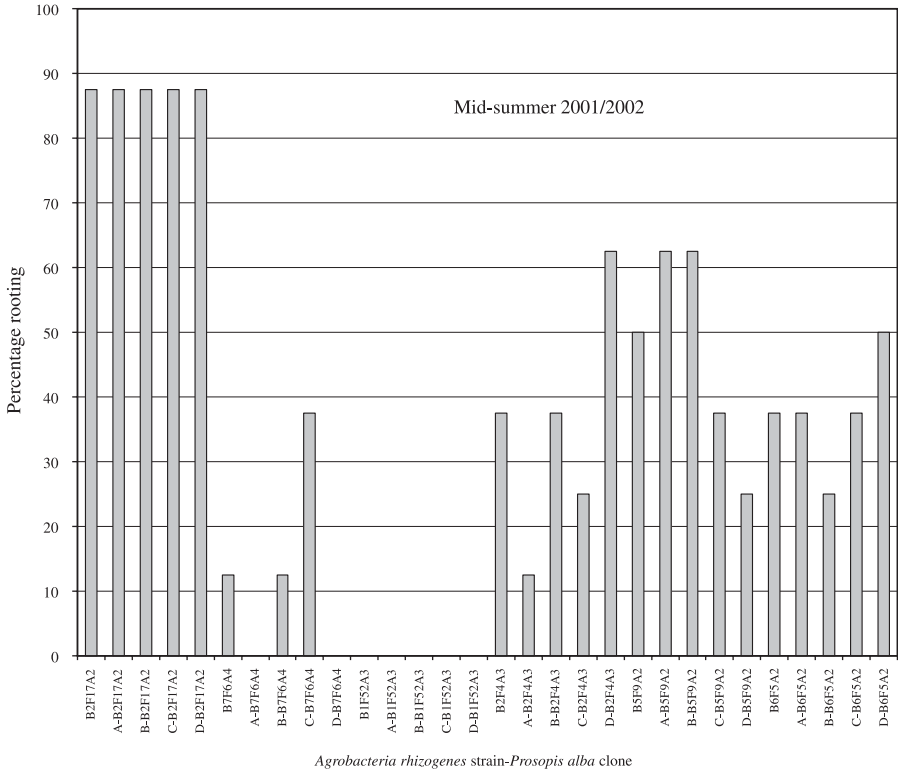
between inoculation dates. There was also no trend on the number of roots per cutting or the length of the longest root per cutting (data not presented).

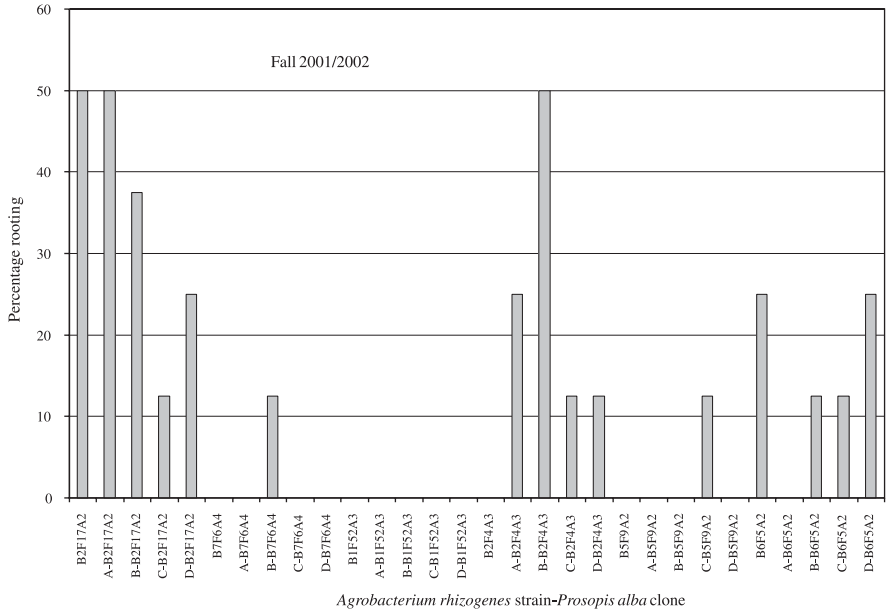
On various occasions NaCl was added to one of the 8001 reservoir tank at a concentration of 1000 mg l^{-1} for one bench but not for the other bench. This addition was made as several reports have indicated that *Fusarium*, a common pathogen in *Prosopis* cuttings, was sensitive to this concentration of NaCl. No differences, positive or negative were observed when this treatment was applied.

In mid summer of 2001/2002 screening trials were initiated with various *Agrobacterium* strains to determine which had the greatest potential for rooting *P. alba* cuttings. From the 12 elite clones available (Felker et al., 2001) six were chosen for experimentation, two that were difficult to root B7F6A4, B1F52A3, three among the easiest to root, B2F17A2, B6F5A2 and B5F9A2 and one that was intermediate B2F4A3. This trial compared a 3 s dip with the hormones alone to hormones plus the various *A. rhizogenes* strains. Two evaluations were made one in mid-summer and the other in late summer/fall with lower air and root temperatures (Fig. 2). The first trial conducted in mid-summer had greater rooting percentages for most all clones than the trial conducted in late summer/fall. In spite of the fact that *A. rhizogenes* has an optimum infection temperature of about 23 °C, the trial with the higher temperature had the greatest rooting percentage. In an experiment not presented we examined the effect of *A. rhizogenes* inoculation at 23 °C in the laboratory sealed in plastic bags under fluorescent lights for times ranging from 1 day to 3 weeks before transfer to the 33 °C rooting bench, but no rooting occurred in this trial with clone B2F4A3. The variation among clones and rooting dates overshadowed any obvious differences in the ability of *A. rhizogenes* strains to induce rooting. To examine *A. rhizogenes*/*P. alba* trials in more detail, we chose *A. rhizogenes* strain B (Tiger 232 from Strobel) as it seemed to be one of the most consistent among clones and rooting dates.

To test the possibility that longer incubation times were required for the *A. rhizogenes* and *Azospirillum* to inoculate the cuttings, we examined (a) no additional bacteria to the hormone solution, (b) the effect of pipetting 0.5 ml of the liquid culture media containing 10^9 bacteria ml^{-1} of *Azospirillum* and/or *A. rhizogenes* at the base of the cutting after it was stuck in the vermiculite on the rooting bench and (c) a 3 s dip that contained the hormones and the both bacteria. As can be seen in Fig. 3 none of the direct high dose inoculations consistently provided additional rooting over the 3 s dip containing hormones and the bacteria. This also did not statistically influence the number of roots per cutting or the length of the longest root per cutting (data not shown).

In contrast to the lack of effect of growth promoting bacteria on rooting, the effect of root temperature had a significant effect on rooting as observed with a comparison identical mist benches with and without bottom heat. Even in mid-summer when maximum air temperatures in the greenhouse were consistently reached 40 °C (Fig. 4) due to the frequent misting, the rooting bench without bottom heat had morning root temperatures were in the low 20s and midday temperatures in the low 30s (Fig. 5). In contrast the bench with heat had morning temperatures in the low 30s and afternoon temperatures between 35 and 40 °C (the hot water circulating


Mid-summer 2001/2002
Percentage rooting
100
90
80
70
60
50
40
30
20
10
0
B2F17A2
A-B2F17A2
B-B2F17A2
C-B2F17A2
D-B2F17A2
B7F6A4
A-B7F6A4
B-B7F6A4
C-B7F6A4
D-B7F6A4
B1F52A3
A-B1F52A3
B-B1F52A3
C-B1F52A3
D-B1F52A3
B2F4A3
A-B2F4A3
B-B2F4A3
C-B2F4A3
D-B2F4A3
B5F9A2
A-B5F9A2
B-B5F9A2
C-B5F9A2
D-B5F9A2
B6F5A2
A-B6F5A2
B-B6F5A2
C-B6F5A2
D-B6F5A2
Agrobacteria rhizogenes strain-Prosopis alba clone


Fall 2001/2002
Percentage rooting
60
50
40
30
20
10
0
B2F17A2
A-B2F17A2
B-B2F17A2
C-B2F17A2
D-B2F17A2
B7F6A4
A-B7F6A4
B-B7F6A4
C-B7F6A4
D-B7F6A4
B1F52A3
A-B1F52A3
B-B1F52A3
C-B1F52A3
D-B1F52A3
B2F4A3
A-B2F4A3
B-B2F4A3
C-B2F4A3
D-B2F4A3
B5F9A2
A-B5F9A2
B-B5F9A2
C-B5F9A2
D-B5F9A2
B6F5A2
A-B6F5A2
B-B6F5A2
C-B6F5A2
D-B6F5A2
Agrobacterium rhizogenes strain-Prosopis alba clone

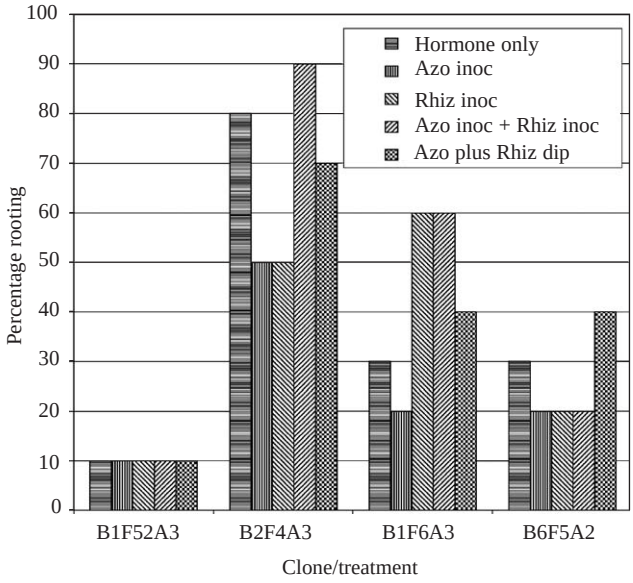


Fig. 3. Influence of various inoculation procedures on rooting of *P. alba* clones. The concentration of the hormone alone solution is described in the text. In the "Inoc" treatments, 0.5 ml of a solution containing 10^9 bacteria ml^{-1} of *Azospirillum* or *A. rhizogenes* strain 232 from Strobel, or both, were pipetted on the stem of the cutting at the location where it entered the vermiculite rooting media. The "Azo + Rhiz dip" treatment consisted of dipping the cuttings for 4 s in a hormone solution that contained about 10^9 bacteria ml^{-1} of *A. rhizogenes* and *Azospirillum*.

pump broke over the December holidays and was not fixed till the New Year resulting in identical temperatures for benches for about 10 days). Owing to this approximate 7 °C increase in rooting temperature for approximately half of the rooting cycle, the rooting percentage (Fig. 6), number of roots (Fig. 7) and length of longest root all increased (Fig. 8). Even *A. rhizogenes* treatments with supposedly optimum temperatures of 23 °C were favoured by these additional temperatures. The higher root temperatures seemed to especially benefit the *Azospirillum* rooting percentages. While the bacterial inoculant combinations were not significantly different from each other for number of roots per cutting or length of longest root per cutting, these parameters were all greatly increased with additional root temperatures.

Fig. 2. Influence of *A. rhizogenes* strain on the rooting percentage of various *Prosopis* clones with bottom heat at two evaluation dates. The treatment with no *A. rhizogenes* strain indicated was inoculated with a 3 s dip of the hormones described in the methods. The *A. rhizogenes* strains were: (A)—ATCC 31798 from Caula Beyl, Plant Science Center, Alabama A&M University, Normal, Alabama; (B)—232 "Tiger" from Gary Strobel, Dept. Plant Pathology, Montana State University, Bozeman, Montana, (C)—ATCC 31799 from Caula Beyl, and (D)—LBA 9402 from N.Curvetto (Universidad Nacional del Sur, Bahia Blanca, Argentina).

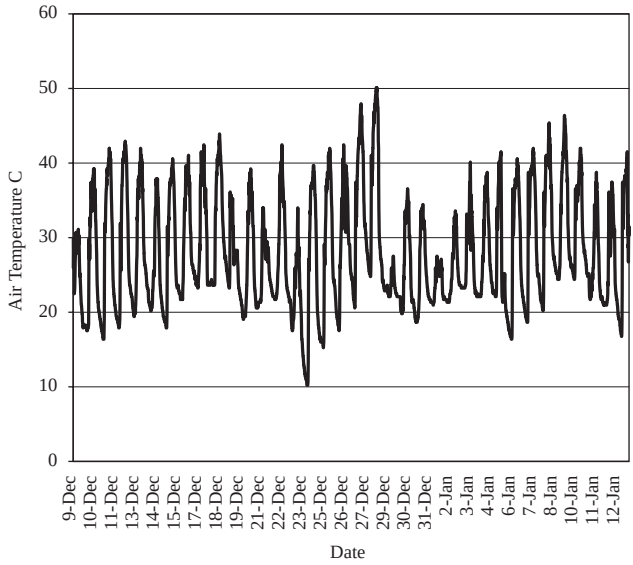


Fig. 4. Air temperature °C measured 1 m above the mist bench in the greenhouse with a HOBO data logger.

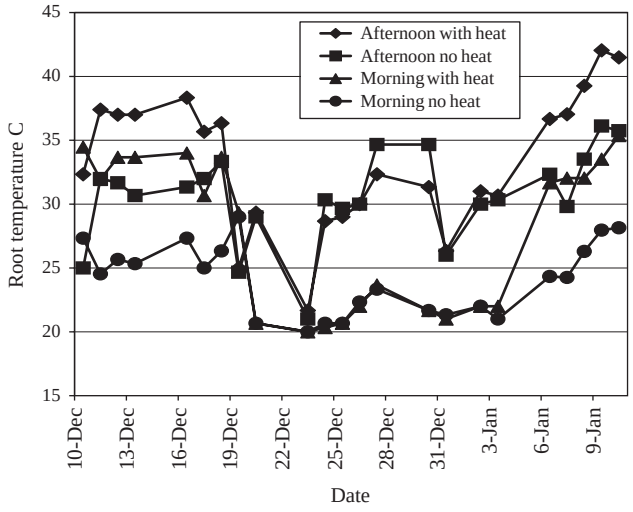


Fig. 5. *P. alba* root temperature as measured with a thermometer in three pots on the mist bench with and without bottom heat at 0830 in the morning or at 1300. From 19 December through 3 January the bottom heat malfunctioned leading to identical temperatures on these dates.

Table 1 compares the mean rooting percentage of these four clones with both *A. rhizogenes* and *Azospirillum* inoculation, with and without bottom heat, to the mean of 21, 1 year old *P. alba* clones selected for tolerance to high salinity evaluated

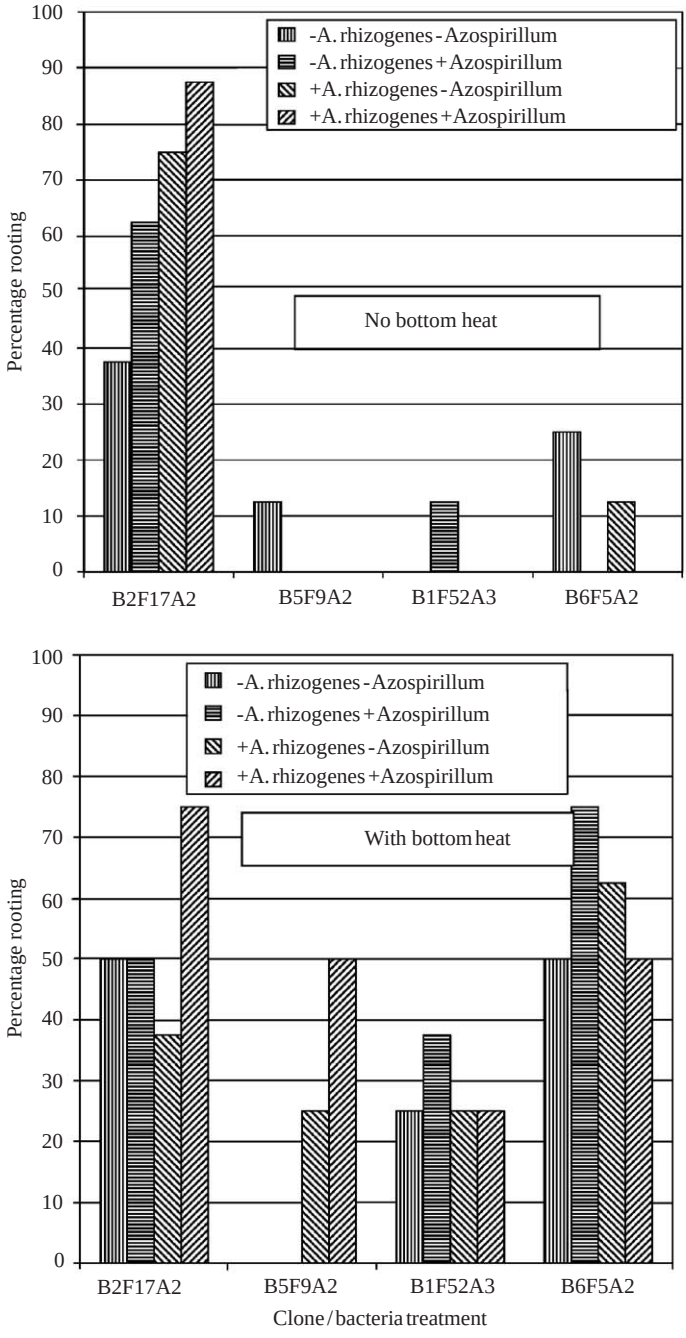


Fig. 6. Rooting percentage of *P. alba* clones when inoculated by a 4 s dip containing combinations of *A. rhizogenes* Tiger 232 or *Azospirillum* from Bellone in Argentina. The treatments were performed on one bench with and one without bottom heat.

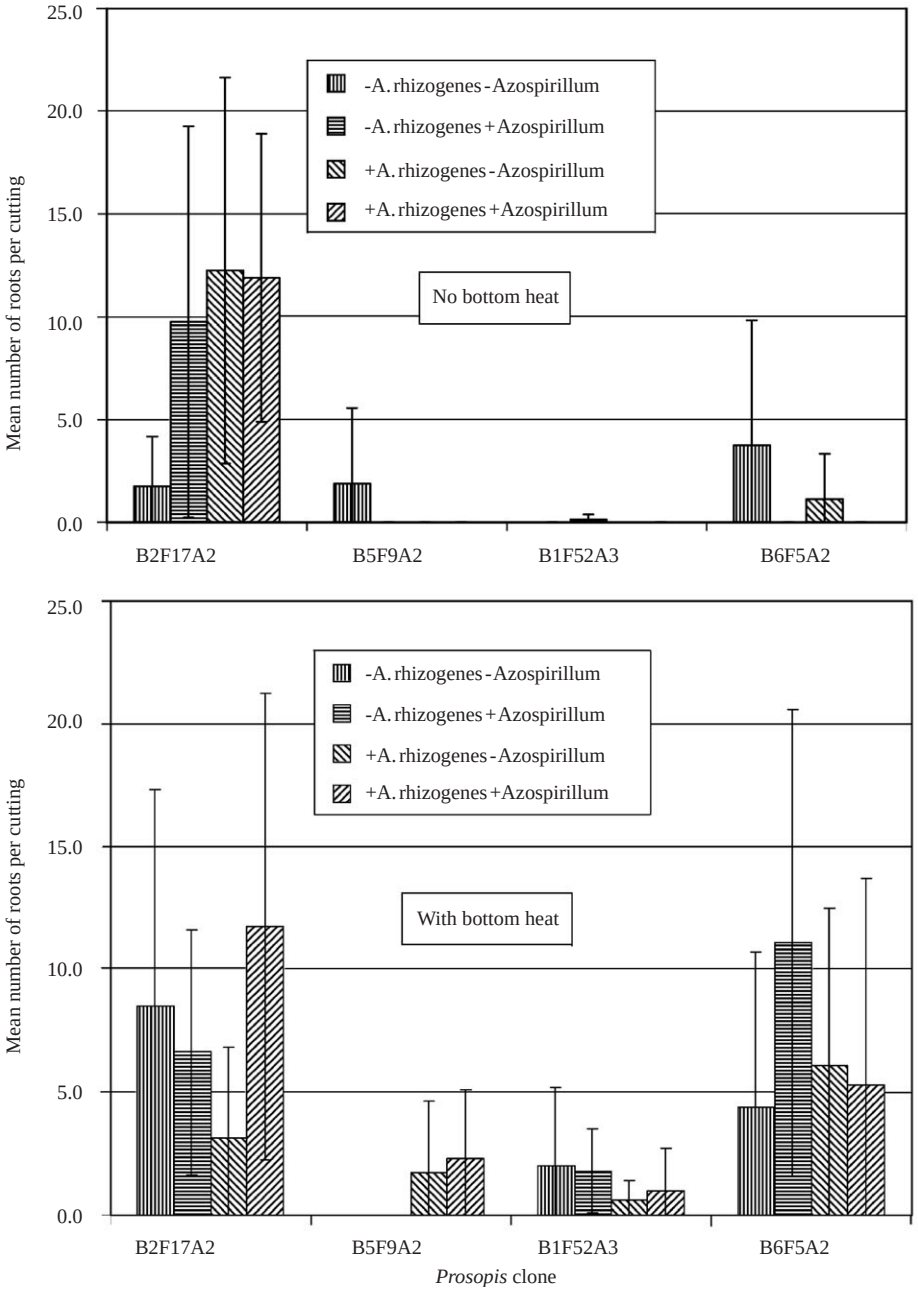


Fig. 7. Mean number of roots per cutting of *P. alba* clones when inoculated by a 4 s dip containing combinations of *A. rhizogenes* Tiger 232 or *Azospirillum* from Bellone in Argentina. The treatments were performed on one bench with and one without bottom heat. The bars are 95% confidence intervals.

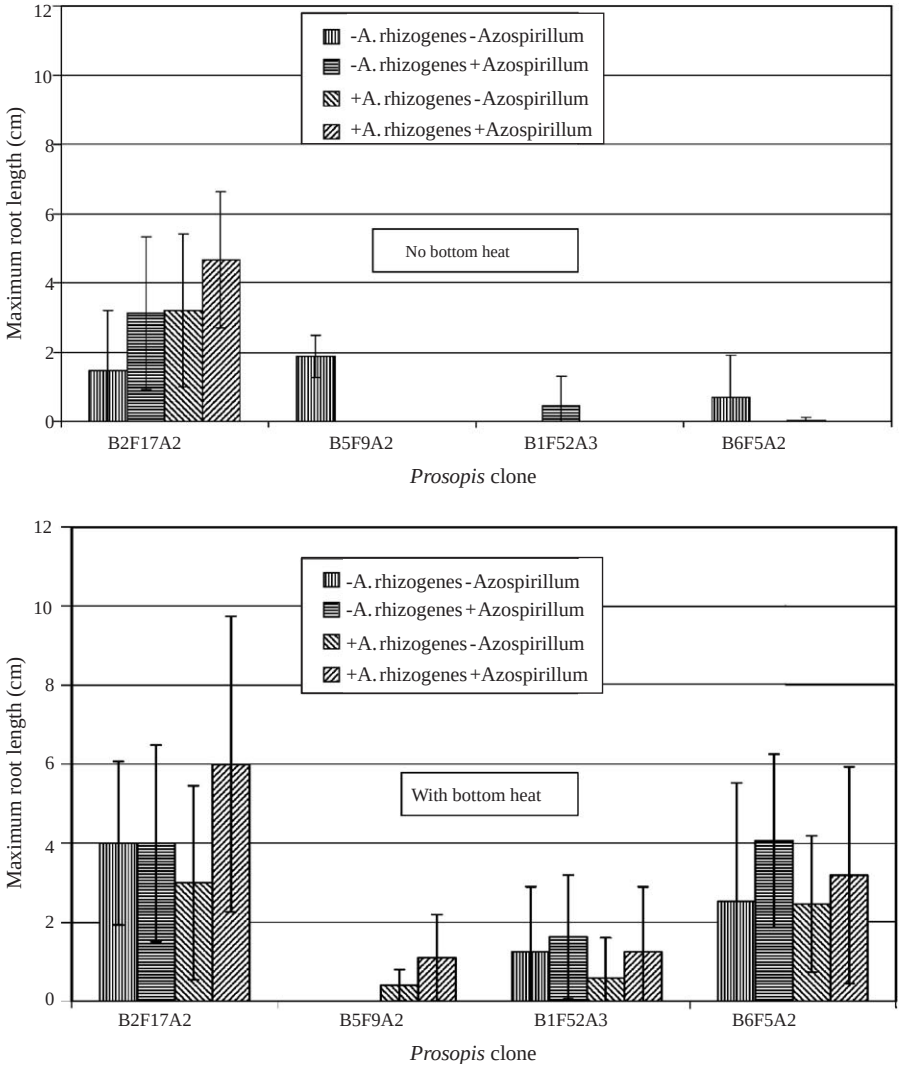


Fig. 8. Mean of maximum root length per cutting of rooting percentage of *P. alba* clones when inoculated by a 4 s dip containing combinations of *A. rhizogenes* Tiger 232 or *Azospirillum* from Bellone in Argentina. The treatments were performed on one bench with and one without bottom heat.

under the same conditions. Both sets of clones show marked improvement in the rooting parameters with the increase of about 7 °C in the rooting temperature. In the case of the younger 1 year old clones, the differences were very close to being significant at the 95% level but due to much higher variation in the older clones, these differences, albeit very large, were not significantly different.

Table 1
Mean root characteristics of 21, 1 year old *P. alba* salt tolerant clones (Velarde et al., 2003) and 4–4 year old *P. alba* multipurpose clones (Felker et al., 2001) with and without bottom heat when treated with rooting hormones and *A. rhizogenes* Tiger 232 and *Azospirillum*

	Rooting percentage, mean (95% C.I)	Number of roots/cutting, mean (95% C.I)	Length longest root, mean (95% C.I)
Mean of 21–1 year old P. alba clones selected for salt tolerance			
With bottom heat	80 (9.5)	30.5(8.5)	3.67(0.57)
Without bottom heat	69.5(12)	23(6.3)	3.00 (0.82)
Mean of 4–4 year old P. alba multipurpose clones			
With bottom heat	50.0(20.0)	5.1(4.9)	2.9(2.2)
Without bottom heat	21.9(42.8)	3.0(5.8)	1.2(2.3)

Five cuttings, with and without bottom heat were evaluated for each the 21 clones and eight cuttings with and without bottom heat were evaluated for the multipurpose clones. Root temperatures are given in Fig. 5.

Although the temperature/bacterial treatments are confounded with a different set of clones from which the cuttings were taken, we have noted a gradual decline in rooting since these stock plants were first grafted in 1998/1999. We believe the lower rooting in the older plants are due to a change in juvenile to mature status of the stock plants.

In spite of the lack of significant treatment differences of the mature stock plants from *Azospirillum* and *A. rhizogenes*, we have never observed such intense root development as occurred in the 1 year old salt tolerant *P. alba* clones. Unfortunately with small 1 m tall stock plants it is not possible to obtain a sufficient number of cuttings to reliably test for treatment effects. The mean of 30 roots per cutting is far greater than the maximum of 12 roots per cutting of Felker and Clark (1981) or 6.7 by Klass et al. (1984) or 5.8 by Klass et al. (1986). An example of one such highly rooted cutting is shown in Fig. 9.

5. Discussion

Despite the great variability in these treatments with few significant differences, we believe we are in a better position to prioritize experiments for further research in rooting cuttings of elite *P. alba* clones.

The first priority would seem to be to optimize the root temperature. As it would be difficult to replicate mist benches with various temperatures, we believe it would be best to develop a mist bench with a gradient in root temperature with 20 °C on one side and 40 °C on the side. Pots could then be aligned in the gradient to find the optimum temperature for rooting. This could be accomplished by using a variable speed pump to adjust the hot water flow in the serpentine so that the exit water was cooled to the ambient mist water temperature.

Fig. 9. Example of one of most developed root systems of cuttings from 1 year old stock plants obtained on the mist bench with hormones, *A. rhizogenes* Tiger 232 and *Azospirillum*.

The second priority would seem to be developing methods to revert the mature stock plants to a more juvenile stage. This might be possible by grafting scions from mature stock plants onto younger rootstock. We have recently reported mini-grafting techniques that are successful on 2 mm diameter seedlings (Ewens and Felker, 2003).

The third priority would seem to be to further evaluate root growth promoting bacteria under the optimum conditions identified above. Among the bacteria we have tested, the *P. aurantiaca*, which was not available in the latter stages of this research, seems most promising. Plant pathologists at the Universidad Nacional de Tucuman have identified five *Fusarium* strains from dying *Prosopis* cuttings based on the hyphae colour (S. Hongn and O. Bain, 2002 pers. comm.) and apparently this pathogen is an important factor in successful rooting of *Prosopis* cuttings. As this *P. aurantiaca* has been shown to have activity against *Fusarium* and as other researchers

(Pal et al., 2001; Paulitz and Belanger, 2001, Rosas et al., 2001) have identified other bacteria such as *P. aurantiaca*, non-pathogenic *Fusarium oxysporum* strain Fo47, *Bacillus* sp. MRF, *Bacillus subtilis* var. *amyloliquefaciens* FZB 24 as having activity against *Fusarium* there is fertile ground for more research on biological control of *Fusarium* in rooting *Prosopis* cuttings.

In spite of the lack of significance of *Azospirillum* alone in these experiments, this micro-organism does have beneficial effects on legumes (Burdman et al., 1997) and there is considerable evidence that *Azospirillum* "is a bacterium that helps other micro-organisms perform better and has an indirect positive effect on plant growth"(Bashan and Holguin, 1997). This kind of "helping action" combined with its ability to release auxins may have contributed to the extensive number of roots we have observed for the first time on *Prosopis* cuttings. Since random sampling of non-inoculated plants found the inoculated strain in controls despite all precautions and since *Azospirillum* have been found in the air year round in an Ohio greenhouse, this will make experiments identifying attributes directly to *Azospirillum* difficult (Bashan and Holguin, 1997).

Use of *A. rhizogenes* to stimulate rooting involves controversy due to the possible transfer of *A. rhizogenes* DNA into the plants. However, the strains we used were non-engineered wild-types, and should some *A. rhizogenes* DNA be inserted into the plant, the cuttings would not be considered genetically modified organisms because this infection could also happen in nature. Thus, when using wild-type strains there is no concern on undeliberate release of GMOs and on public opinion. The only problem might be the spread of pathogenic bacteria into the environment.

It is encouraging to note that this bacterium stimulated rooting of *Pinus monticola, Pinus banksiana* and *Larix laricina* (McAfee et al., 1993) and *Pinus sylvestris* (Aronen et al., 1996) without inserting *A. rhizogenes* DNA into the plants. Thus, this should even further decrease criticism about release of plants with modified DNA into the environment.

Should the social/legal issues related to release of plants transformed to include the *RolB* gene be resolved, more intensive efforts to stimulate transformation could be attempted. For example Villalobos-Amador et al. (2002) had low rooting percentage of *Pinus maximartinezii* and *P. pinceana* in vitro with *A. rhizogenes* until they included acetosyringone in the culture media. This signal molecule is produced on wounding plants and triggers the activation of virulence genes needed for the transfer of DNA into the plant cell. Other workers found that incorporation of opines, which are produced and utilized by the *A. rhizogenes* as a food source, stimulated a 2–10-fold increase in transformation (Veluthambi et al., 1989) by the related bacterium *A. tumefaciens*. The most promising report of Zhu et al. (2001) obtained an increase in rooting from 0% to 83% to 100% in difficult to root apples clones, by genetically transforming the plants by incorporation of the *rolB* gene.

If it were necessary to co-culture every desired plant with *A. rhizogenes* in tissue culture this would lead to high costs for planting stocks. Haggman and Aronen (2000) pointed out "Rooting in vivo (as opposed to in vitro tissue culture) is more or less a prerequisite for commercial use of this technique [*A. rhizogenes*]". We would like to add that for commercial use, the technique must not only be successful at the

greenhouse/nursery level, but it must be successful from material taken stock plants derived from elite trees from progeny trials or even-aged stands. Development of techniques for asexual propagation from several-day-old seedlings suffers from the fact that juvenile tissue is always easier to root that mature tissue, and clonal material derived from outcrossed seed of unproven performance has little utility for plantations.

As opposed to other research areas where we have worked, such as fertility, effects of salinity stress on growth, nitrogen fixation, plantation establishment-rooting of cutting research has greater variability and much fewer experiments that have significantly different treatment effects. Owing to the great variability in seedlings resulting from this obligately outcrossed species, great genetic gains can be achieved with multi-purpose clones (Felker et al., 2001; Alban et al., 2002) that in turn can lead to considerably shorter rotation lengths and an almost doubling in the internal rates of return for plantations (Felker and Guevara, 2003). Thus, it is important for both researchers and funding agencies to have patience in the quest for commercially viable techniques for asexual reproduction of elite *P. alba* clones.

In this survey of environmental and plant growth promoting bacteria effecting rooting of *P. alba* cuttings, perhaps the factor with the greatest influence was the effect of root temperature on rooting cuttings. However, as only two temperatures were examined, detailed research is needed to determine the optimal rooting temperature. The finding that the mean rooting percentage for 21, 1-year old *P. alba* stock plants was 80% vs. 50% for four, 4 year old stock plants suggests a decline rooting with stock plant age. This in turn suggests that trials to rejuvenate the stock plants, perhaps by regrafting onto younger rootstock should be conducted. Less clear effects on rooting were observed with root promoting bacteria. However, the greater root number per cutting observed here (30 per cutting) than reported in previous studies (6–12) suggests a synergistic effect has occurred that is difficult to demonstrate statistically. In light of the frequency of *Fusarium* on dying cuttings, the literature demonstration of the effect of *Pseudomonas* as a biocontrol agent of *Fusarium* and the stimulating effect of *P. aurantiaca* on some of the *P. alba* clones, this Rhizobacteria would seem to have the greatest potential for further research. While *A. rhizogenes* may have an effect, the liability of negative public opinion with use of plants possibly containing *A. rhizogenes* genes would indicate a lower priority for these bacteria. After the optimum root temperature is determined, future trials should reexamine various *Azospirillum* strains for their influence on rooting in conjunction with other bacteria. Should it be possible to maintain the clones in a juvenile state, the treatment with *Azospirillum*, *A. rhizogenes*, hormones and 35 °C root temperature will provide commercially acceptable rooting successes of 80% rooting with 30 roots per cutting.

Acknowledgements

The financial assistance of the US Department of Agriculture Cooperative Agreement 58-3148-8-041 and of the Forestry Experiment Station of the Catholic

University of Santiago del Estero and valuable discussions with Atilio Castagnaro and Juan Carlos Diaz Ricci are gratefully acknowledged.

References

Alban, L., Matorel, M., Romero, J., Grados, N., Cruz, G., Felker, P., 2002. Cloning of elite, multipurpose trees of the *Prosopis juliflora/pallida* complex in Piura, Peru. Agroforestry Systems 54, 173–182.

Aronen, T.S., Haggman, H.M., Salonen, M., 1996. Rooting of scots pine fascicular shoots by *Agrobacterium rhizogenes*. Forest Genetics 3, 13–22.

Bashan, Y., Holguin, G., 1997. *Azospirillum*-plant relations: environmental and physiological advances (1990–1996). Canadian Journal of Microbiology 43, 103–121.

Burdman, S., Vedder, D., German, M., Itzigsohn, R., Kigel, J., Jurkevitch, E., Okon, Y., 1997. Legume crop yield promotion by inoculation with *Azospirillum*. In: Elmerich, C., Kondorosi, A., Newton, W.E. (Eds.), Biological Nitrogen Fixation for the 21st Century. Kluwer Academic Publishers, Dordrecht, pp. 609–612.

Caro, L.A., Santecchia, N., Marinangeli, P.A., Curvetto, N.R., Hernández, L.F., 2003. *Agrobacterium rhizogenes* vs auxinic induction for in vitro rhizogenesis of Prosopis chilensis and Nothofagus alpina. Biocell 27 (3), 311–318.

DeSouza, S., Felker, P., 1986. The influence of stock plant fertilization on tissue concentrations of N, P, and carbohydrates and the rooting of *Prosopis alba* cuttings. Forest Ecology and Management 16, 181–190.

Ewens, M., Felker, P., 2003. The potential of mini-grafting for large scale commercial production of *Prosopis alba* clones. Journal of Arid Environments 55, 379–387.

Felker, P., 2005. Unusual physiological properties of the arid adapted tree legume *Prosopis* and their applications in developing countries. In: De la Barrera E. (Ed.), Biophysical Plant Ecology: Perspectives and Trends. Universal Publishers, in press.

Felker, P., Clark, P.R., 1981. Rooting of mesquite (*Prosopis*) cuttings. Journal of Range Management 34, 466–468.

Felker, P., Guevara, J.C., 2003. Potential of commercial hardwood forestry plantations in arid lands-An economic analyses of *Prosopis* lumber production in Argentina and the United States. Forest Ecology and Management 186, 271–286.

Felker, P., Lopez, C., Soulier, C., Ochoa, J., Abdala, R., Ewens, M., 2001. Genetic evaluation of *Prosopis alba* (algarrobo) in Argentina for cloning elite trees. Agroforestry Systems 53 (1), 65–76.

Haggman, H.M., Aronen, T.S., 2000. *Agrobacterium rhizogenes* for rooting recalcitrant woody plants. In: , Molecular Biology of Woody Plants, vol 2. Kluwer Academic Publishers, The Netherlands, pp. 47–78.

Hatta, M., Beyl, C.A., Garton, S., Diner, A.M., 1996. Induction of roots on jujube softwood cuttings using *Agrobacterium rhizogenes*. Journal of Horticultural Science 71 (6), 881–886.

Klass, S., Bingham, R.L., Finkner-Templeman, L., Felker, P., 1984. Optimizing the environment for rooting cuttings of highly productive clones of *Prosopis alba* (mesquite/algarrobo). Journal of Horticultural Science 60, 275–284.

Klass, S., Wright, J., Felker, P., 1986. Influence of Auxins, thiamine, and fungal drenches on the rooting of *Prosopis alba* clone B_2V_{50} cuttings. Journal of Horticultural Science 60, 97–100.

Leakey, R.R.B., Mesen, J.F., Tchoundjeu, Z., Longman, K.A., Dick, J.McP., Newton, A., Matin, A., Grace, J., Munro, R.C., Muthoka, P.N., 1990. Low technology techniques for the vegetative propagation of tropical trees. Commonwealth Forestry Review 69, 247–257.

McAfee, B.J., White, E.E., Pelcher, L.E., Lapp, M.S., 1993. Root induction in pine (*Pinus*) and larch (*Larix*) spp using *Agrobacterium rhizogenes*. Plant Cell Tissue and Organ Culture 34, 53–62.

Moore, L., Warren, G., Strobel, G., 1979. Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. Plasmid 2, 617–626.

Pal, K.K., Tilak, V.B.R., Saxena, A.K., Dey, R., Singh, C.S., 2001. Suppression of maize root diseases caused by *Macrophomia phaseolina*, *Fusarium moniliforme* and *Fusarium graminaerum* caused by plant growth promoting rhizobacteria. Microbiological Research 156, 209–223.

Pasiecznik, N.M., Felker, P., Harris, P.J.C., Harsh, L.N., Cruz, G., Tewari, J.C., Cadoret, K., Maldonado, L.J., 2001. The *Prosopis juliflora–Prosopis pallida* complex. A Monograph, HDRA, Coventry, UK, 162pp.

Paulitz, T.C., Belanger, R.R., 2001. Biological control in greenhouse systems. Annual Review of Phytopathology 39, 103–133.

Rosas, S., Altamirano, F., Schroder, E., Correa, N., 2001. In vitro biocontrol activity of *Pseudomonas aurantiaca*. International Journal of Experimental Botany PHYTON, 203–209.

Strobel, G.A., Nachimas, A., 1985. *Agrobacterium rhizogenes* promotes the initial growth of bare root stock almond. Journal General Microbiology 131, 1245–1249.

Velarde, M., Felker, P., Degano, C., 2003. Evaluation of Argentine and Peruvian *Prosopis* germplasm for growth at seawater salinities. Journal of Arid Environments 55, 515–531.

Veluthambi, K., Krishnan, M., Gould, J.H., Smith, R.H., Gelvin, S.B., 1989. Opines stimulate induction of the vir genes of the *Agrobacterium tumefaciens* Ti plasmid. Journal of Bacteriology (7), 3696–3703.

Villalobos-Amador, E., Rodriguez-Hernandez, G., Perez-Molphe-Balch, E., 2002. Organogenesis and *Agrobacterium rhizogenes*-induced rooting in *Pinus maximartinezii* and *P. pinceana* Gordon. Plant Cell Reports 20, 779–785.

Whiteman, K., Him, R., Scheffer, R.J., Strobel, G.A., 1988. Factors influencing root formation in dicots by *Agrobacterium rhizogenes*. Canadian Journal of Botany 66, 642–644.

Wojtusik, T., Boyd, M.T., Felker, P., 1994. Effect of different media on vegetative propagation of *Prosopis juliflora* under solar powered mist. Forest Ecology and Management 67, 267–271.

Zhu, L.H., Ahlman, A., Li, X.Y., Welander, M., 2001. Integration of the rolA gene into the genome of the vigorous apple rootstock A2 reduced plant height and shortened internodes. Journal of Horticultural Science and Biotechnology 76, 758–763.