Bulgarian Journal of Agricultural Science, 23 (No 4) 2017, 572–577
Agricultural Academy

AGROBACTERIUM RHIZOGENES – MEDIATED HAIRY ROOT INDUCTION IN GARLIC

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Abstract

Moradi, F., M. Z. Mehrjerdi and K. Vahdati, 2017. *Agrobacterium rhizogenes* – mediated hairy root induction in garlic. *Bulg. J. Agric. Sci.*, 23 (4): 527–577

Three factors including *Agrobacterium rhizogenes* strains (A4, A7 and ATCC15834), genotypes (Gorgan and Ramhormoz) and explant types (stem disc meristem, shoot apical meristem and root apical meristem) were conducted to study the effects on hairy root induction of garlic. Our results showed that *A. rhizogenes* strains and explants types had significant effect on root induction frequency (RIF), while the genotypes hadn't effect on the RIF. The frequency of root induction was up to 23.3%. Some the combination of three factors could induce hairy roots.The results of PCR analysis showed that expected fragments with length of 780bp corresponding to the *rolB* gene was amplified from hairy root cultures. To our knowledge, this study was the first comprehensive evaluation of garlic hairy root induction.

Key words: Agrobacterium rhizogenes; explants; garlic; genotype; hairy root

Introduction

Garlic (*Allium sativum* L.) is one of the most important vegetable belongs to the *Alliaceae* family that has been widely utilized as seasoning, flavoring, culinary and in herbal remedies (Suleria et al*.,* 2015). It is contains some amounts of the three energy generating nutrients and significant amounts of minerals- including: manganese, calcium, phosphorus, copper, sodium, selenium, the amino acid tryptophan, and vitamins B1, B6 and C (El-Sabban, 2015). It also possesses a number of phytochemicals compounds, such as alliin, methiin and S-allylcysteine (Nicastro et al., 2015). The main source of garlic essential oils is it's bulbs with the essential oil yield about 1% per 100 g of dried samples (Dziri et al., 2014). One of the main problems to produce secondary metabolism in plants is being long times of production. For garlic it takes more than seven month. Hairy root cultures have been advocated for the production of plant secondary metabolites in a short time (Allan et al*.*, 2002). They exhibit rapid growth and do not require plant growth regulators (Srivastava and Srivastava, 2007). *Agrobacterium rhizogenes* is a gram-negative soil-borne bacterium that is able to induce hairy roots. In the present study, for hairy root induction, we evaluated two garlic genotypes and three *A. rhizogenes* strains. According to molecular analysis, these genotypes have a high distance from each other and is presented in different groups (Vafaei et al*.*, 2009). Therefore both of them are important sources of germplasm for future breeding programs. Three *A. rhizogenes* strains, ATCC15834, A4 and A7 were studied in the study. The strains were previously used in genetic transformation in different plants. ATCC15834 and A4 strains widely used in hairy root induction in many other plant species. Sharafi et al. (2014) reported a new protocol for in vitro plant regeneration a genetically transformed root induction in *Artemisia aucheri* by four bacterial strains, ATCC15834, A4, A13 and MSU440 (Sharafi et al*.*, 2014). Poerwanto et al. (2007) studied 11 strains of *A. rhizogenes* bacterium for inoculation of mangosteen seedling root, that two of them were ATCC15834 and A4 (Poerwanto et al., 2007). These two strains were used on the induction of transgenic hairy roots in 14 *Vitis* species (Jittayasothorn et al*.*, 2015). A4 and A7 strains were used in hairy roots formation in four *Solanaceae* species (Shakeran et al., 2014). In this study, we studied the influences of two genotypes, includ-

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ing Gorgan and Ramhormoz genotypes, three *A. rhizogenes* strains including ATCC15834, A4 and A7 and three plant tissue types including shoot apical meristem, root apical meristem and stem disc meristem on garlic hairy root induction. To our knowledge, this study was the first comprehensive evaluation of garlic hairy root induction.

Materials and Methods

Plant materials

The bulbs of two genotypes including Gorgan and Ramhormoz were selected. Garlic cloves were separated from each other and then papery skins were removed. The cloves were washed by sterile distilled water (SDW) then were disinfection with 1.5% sodium hypochlorite for 30 min and washed by 70% ethanol for 10 min finally washed three times with SDW. Subsequently the cloves were transferred to laminar hood in order to explants isolation. In the present study three explant types including shoot apical meristem, root apical meristem and stem disc meristem were selected. Shoot apical meristem and stem disc meristem explants were isolated from the cloves whereas to obtain root apical meristem explant some of cloves were cultured in MS medium. When the cloves roots were at least 2 cm, the root apical meristem was isolated.

Bacteria

Three *A. rhizogenes* including 15834, A4 and A7 were obtained from Faculty of New Science and Technology University of Isfahan. The strains were cultured on LB medium supplemented with 50 mg l^{-1} rifampicin and grown at 28°C until suspension concentration reached 0.6-0.8 OD_{600}.The medium was centrifugation (10 min at 3500 rpm) and the supernatant was discarded. Then bacteria were collected and resuspended in 10 ml of fresh MS liquid medium supplemented with 100 µM acetosyringone.

Transformation of garlic

Explants were submerged in the suspension of all strains for 10 min. After it explants were transferred to MS medium in darkness for 48 h at 25ºC. The explants were then transferred to new MS medium containing 250 mg/l cefotaxime. Subsequent subcultures were on same medium every 2 weeks. Root induction frequency was measured as following formula:

$$\text{Root induction frequency} = \frac{\text{number of explants with induced roots}}{\text{total number of explants used for transformation}}$$

PCR analysis

Polymerase Chain Reaction (PCR) method was used to detect *rolB* gene in established "hairy" roots. DNA of non-transformed roots was used as the negative control. DNA isolation was performed using the CTAB method. DNA from the transformed hairy roots and the control non-transformed roots was used for PCR. The *rolB* gene of *A. rhizogenes* was used as the target for the PCR confirmation study. The primer sequence and the conditions for amplification of the *rolB* are shown in Table 1 and Table 2, respectively.

Table 2
PCR reaction conditions used for confirmation of *rolB* gene in HRs culture of *Allium sativum*

PCR step	Temperature (ºC)	Time	No. of cycles
Initial denaturation	94	5 min	1
Denaturation	94	1 min	35
Annealing	53	1 min	-
Extension	72	1 min	-
Final extension	72	5 min	1

In order to assessment of influence of genotypes, *A. rhizogenes* strains and explant types, the experiments were arranged as a 2×3×3 factorial in a completely randomized design with three replicates. Analysis of variance was performed on the data and means were separated using Duncan's multiple range test.

Results and Discussion

Result of ANOVA

The analysis of variance showed that *A. rhizogenes* strains and explant types had significant effect on root induction frequency ($P \leq 0.01$), whereas garlic genotypes didn't have significant effect on the RIF (Table 3). Also all factors interactions had significant effect on the RIF except for strain-genotype-explant interaction. Interaction between genotype with strain and genotype with explant were significant at 5% level but interaction between strain and explant was significant at 1%.

Table 1
Primer used to confirm the presence of transgenes in *Allium sativum* "hairy" roots

Gene	Sequence	Size of amplified fragment	Annealing temperature
rolB	5'-ATGGATCCCAAATTGCTATTCCCCACGA-3' 5'-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3'	780 bp	53ºC

Table 3
Analysis of variance of effects of different factors on root induction frequency

Source of variation	df	Mean Square
Strain	2	0.0067**
Genotype	1	0.0007n.s
Explant	2	0.081**
S*G	2	0.0016*
S*E	4	0.0022**
G*E	2	0.0016*
S*G*E	4	0.0011n.s
Error	36	0.0004
CV (%)	2.9	-

n.s: Non significance.**and*indicates significance at 1% and 5% levels respectively.

Effects of different A. rhizogenes strains on the RIF

The strains, 15834, A4 and A7 showed different capabilities in generation of root induction frequency on in vitro garlic plants. Similar results were reported in many previous studied on different plants (Grabkowska et al., 2010; Varghese et al., 2014; Bivadi et al., 2014; Wahyuni et al., 2015). The result of Duncan test indicated that the A4 strain had the highest RIF (11%) followed as A7 (6.1%) and 15834 (5.5%) strains (Figure 1). The A7 and 15834 strains didn't have different significant with each other. Compared with strain A4, two others strains showed a lower efficiency for production of root induction frequency. In many studied two strains A4 and 15834 were used to root induction in different plants (Varghese et al., 2014; Krol et al., 2014; Basu et al., 2015). Bivadi et al. (2014) and Jittayasothorn et al. (2015) reported that 15834 strain had more effective to root hair induction than A4 strain that is contrast with our results (Bivadi et al., 2014; Jittayasothorn et al., 2015).

In order to confirmation of root transformation, PCR analysis of hairy root DNA was used. The results of PCR analysis showed that expected fragments with length of 780 bp corresponding to the *rolB* gene was amplified from hairy root cultures but not from control plant (normal roots) (Figure 2 and Figure 3).

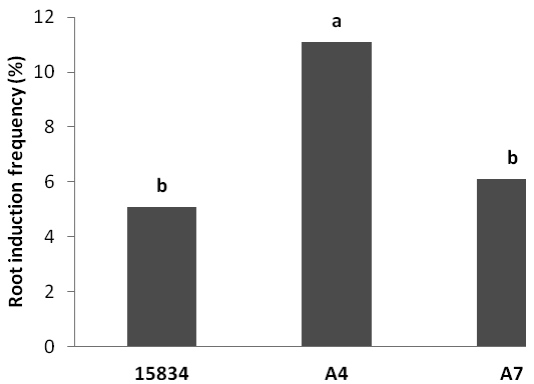

Fig. 1. Effects of different *A. Rhizogenes* strains on root induction frequency (%) in garlic

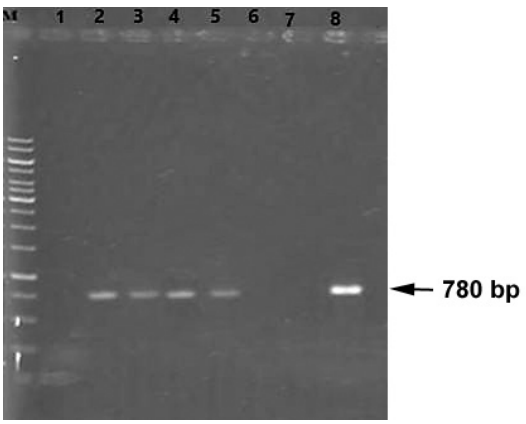

Fig. 2. PCR analysis for hairy roots lines of garlic using the *rolB* gene specific primers. M: 1kb DNA ladder, 1: Non-DNA template PCR reaction, 2-5: transgenic hairy root lines, 6-7: Non transgenic roots, 8: Ri plasmid from *A. rhizogenes*

Effects of garlic genotypes on the RIF

In the present study we selected two garlic genotypes including Gorgan and Ramhormoz genotypes. The genotypes were selected according to the allicin properties. Gorgan and Ramhormoz genotypes had high and low levels of allicin, respectively. The result of ANOVA showed that there was not different significant between genotypes. However the Gorgan genotype (8.1%) had more respond to the RIF than Ramhormoz genotype (7%) (Figure 4). The genotypes almost had equal the RIF, indicated that the level of allicin didn't have any affected on the RIF. However Vafaei et al. (2009) reported that the genotypes had a high distance from each other and were presented in different groups (Vafaei et al., 2009). Our genotypes didn't have variation in the RIF but in many plants, genotypes showed a high level of root induction variation such as soybean (Weber and Bodanese-Zanettini, 2011; Savka et al., 1990), *Vitis* species (Jittayasothorn et al., 2015), tomato (Peres et al., 2001) and potato (De Vries-Uijtewaal et al., 1988).

Effects of different explant types on the RIF

In the present study, wounded shoot apical meristem, root apical meristem and stem disc meristem of in vitro garlic genotypes were inoculated with *A. rhizogenes* to determine effects of different explants on the RIF. Among the three explants root apical meristem didn't have any hairy root induc-

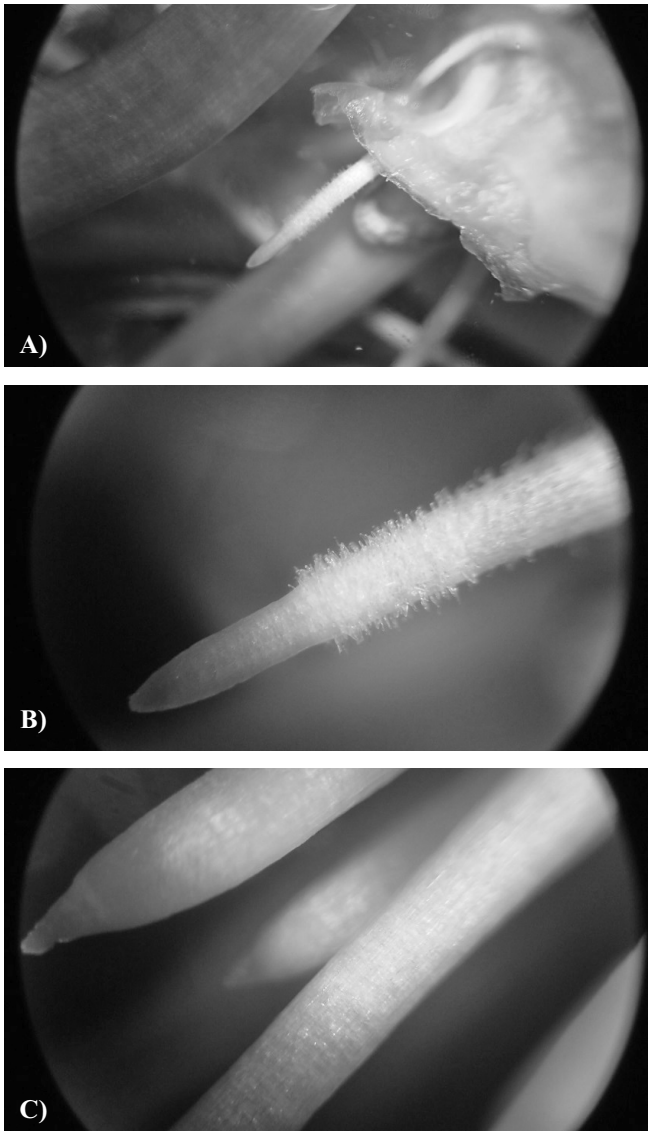

Fig. 3. The transformed hairy roots (A) and normal roots (B) of garlic

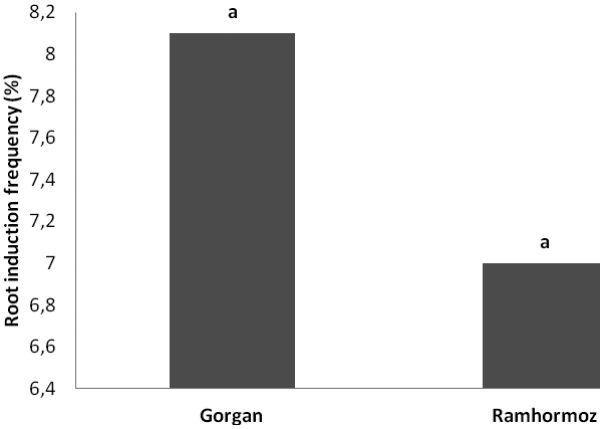

Fig. 4. Effects of two garlic genotypes on root induction frequency (%) in garlic

tion. The result of Duncan test indicated that stem disc meristem explant had the highest RIF (19.4%). Also shoot apical meristem produced the RIF about 3.3% (Figure 5). Our results revealed that for hairy root induction stem disc meristem explant was more productive the RIF than shoot apical meristem and root apical meristem on in vitro garlic genotypes. A contrast observation was reported by Grąbkowska et al. (2010) that high frequencies of transformation were obtained from stem segments carrying intercalary meristems (53.6%) and shoot apical meristems (40.8%) in four explant types tested for hairy root induction in *Harpagophytum procumbens* (Grabkowska et al., 2010). Gangopadhyay et al. (2010) studied effects of three strains of *A.rhizogenes* and two explants including leaf and stem on hairy root induction in *Plumbago indica*(Gangopadhyay et al., 2010). Authors reported that the hairy root generated from stem explants infected with all strains was not found.

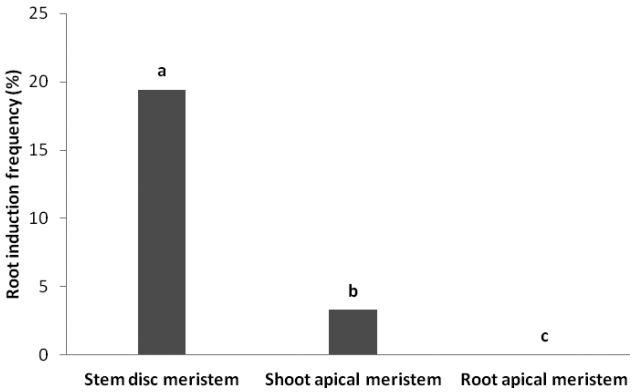

Fig. 5. Effects of different explant types on root induction frequency (%) in garlic

Effects of different interaction among factors studied on the RIF

According to the ANOVA results, all interactions were significant except for strain-genotype-explant interaction (Table 3). In interaction of strain-genotype, interaction of A4 strain with Gorgan genotype had the highest RIF (13.3%) (Figure 6). All others strain-genotype interactions didn't have any significant with each other. Our results confirmed many the previous reported that both A4 and 15834 strains can induce hairy roots in different plant genotypes, such as *Vitis* species (Jittayasothorn et al., 2015), *Festuca rubra* (Krol et al., 2014), *Salvia miltiorrhi-*

za (Tan et al., 2014) and four species of *Solanaceae* (Shakeran et al. 2014). Shakeran et al. (2014) used of these *A. rhizogenes* strains including to hairy root induction in four *Solanaceae* species. Authors reported that hairy root induction was generated in all strains-species interaction (Shakeran et al., 2014).

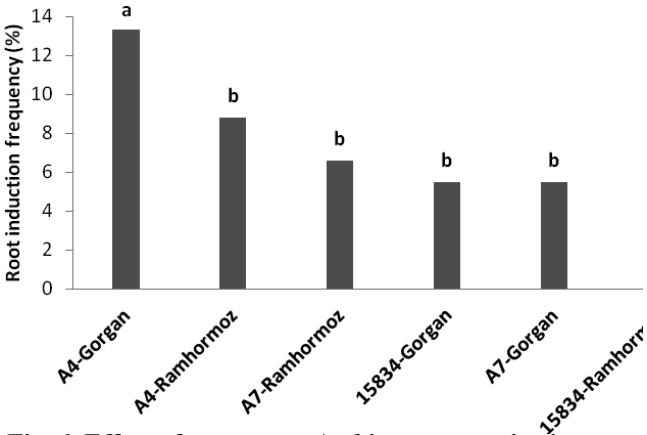

Fig. 6. Effect of genotypes-*A. rhizogenes* strains interaction on root induction frequency (%) in garlic

In interaction of genotype-explant, interaction of stem disc meristem with both genotypes had the highest RIF (18.8 and 20.1% in Gorgan and Ramhormoz genotypes, respectively), whereas the RIF was not observed in interaction of root apical meristem explant with both genotypes (Figure 7). Among three explant types for hairy root induction stem disc meristem explant was found to be best explant. To our knowledge so far is not any report to study interaction of genotype and explants to hairy root induction in plants. However Hoque and Mansfield (2004) studied effect of explant and genotype on callus induction from root-derived callus of *Indica* rice genotypes. Authors reported that regeneration responses were found to be affected by the genotype and age of root explants (Hoque and Mansfield, 2004). A4 with stem disc meristem explant treatment had the highest RIF in interaction effect of strain-explant (23.3%) (Figure 8).

Among three explants stem disc meristem explant only with the three strains generated the RIF and other strain-explant treatments except for A4- shoot apical meristem hadn't produced the RIF. Bansal et al. (2014) studied the influence of *A. Rhizogenes* strains (including A4, R1000, SA79, MTCC 532 and MTCC 2364) and different explants on hairy root induction in *Bacopa monnieri* (L.). Among all the five strains that were used, transformation frequency from leaf explants was higher than inter nodal segments and maximum root induction was observed from leaf explants infected with strain SA79 (Bansal et al., 2014). Giri et al. (2001) reported influence of different A. strains on hairy root induction in *Artemisia annua* using shoot-tip meristem explants. All strains were able to infect the explants (Giri

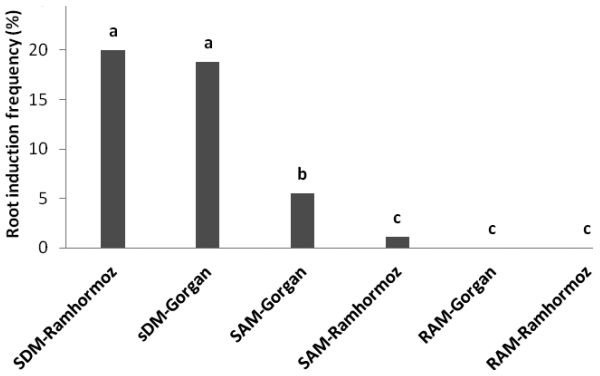

Fig. 7. Effect of genotypes- explant types interaction on root induction frequency (%) in garlic (SDM: Stem disc meristem; SAM: Shoot apical meristem; RAM: Root apical meristem)

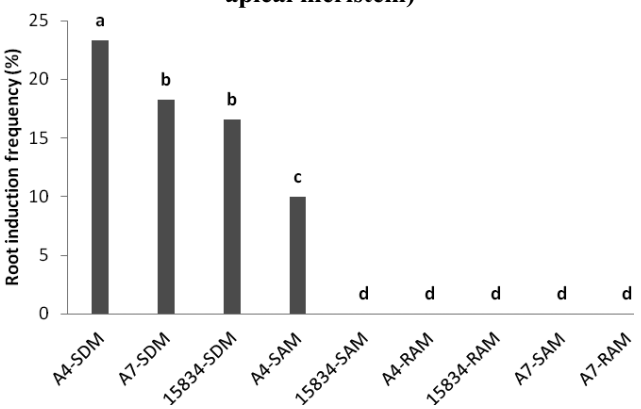

Fig. 8. Effect of *A. rhizogenes* strain-explant type interaction on root induction frequency (%) in garlic (SDM: Stem disc meristem; SAM: Shoot apical meristem; RAM: Root apical meristem)

et al. 2001). In our result only A4 strain was able to infect the shoot apical meristem explant. Also root apical meristem was resistant to infect by the three strains.

Conclusions

For first time we used *A. rhizogenes* strains, genotypes and explant types to induce hairy root formation from *Allium sativum*. Our results demonstrated that *A. rhizogenes* strains and explant types had significant effect on the hairy roots induction in garlic. On the other hand, the genotypes had not effect on the hairy roots induction. Also all interaction effects of the factors studied had significant effect on the RIF (except for tripartite interaction effect). The frequency of root induction was up to 23.3% and it was differ in the case of the factors used. Our results prepared a comprehensive light on the generation of transgenic hairy root in garlic plant.

Acknowledgments

The authors are grateful to Dr. Keyhanfar and Dr. Hasanloo for providing the *A. rhizogenes* strains used in this work.

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Received April, 23, 2017; accepted for printing June, 9, 2017