ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Invitro Callus induction and Root regeneration through the mediation of *Agrobacterium rhizogenes* in *Punica granatum*

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Manuscript Info

Manuscript History:

Received: 10 March 2015
Final Accepted: 18 April 2015
Published Online: May 2015

Key words:

Punica granatum, callus, auxins, *A.rhizogenes*, etc

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Abstract

In *Punica granatum* highly efficient and reproducible callus and root induction has been developed through *A. rhizogenes* by using juvenile segment as explant source. One month old calli were treated with *A. rhizogenes* for root induction in MS medium without auxins. The best roots were derived from callus of pomegranate through the mediation of *A.rhizogenes*. This protocol opens up prospects for using biotechnological approaches for pomegranate improvement.

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INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family punicaceae, which comprises only one genus and two species, other being *Punica protopunica* (Evreinoff 1949; Zukovskij 1950; Moriguchi *et al.*, 1987; Guarino *et al.*, 1990; Jbir *et al.*, 2008). Pomegranate is native to Iran, Afganisthan, Baluchistan and Himalayas in Northern India. This is popularly known as anar, Dalimba in India and is one of the oldest favourite table fruits of tropical and subtropical regions of the worlds (Pekmezci and Erkan 2003). Pomegranate is exploited for nutritional value of its fruit, the medicinal properties of different parts of the tree, and its use as an ornamental (Parmar and Kaushal, 1982; Naovi *et al.*, 1991; Jayesh and Kumar 2004; Johanningsmeier and Harris 2011). The fruit is a rich source of minerals, vitamins, antioxidant polyphenols, potassium and fair source of iron. India is second largest producer of pomegranate with a production of 8.07 lakh tons.

To get true to type planting material Pomegranate is commercially propagated by stem cuttings (Hardwood cutting) or by air layering. However it has several limitations like low success, very slow propagation method and new plant requires one year for establishment. This results in non-availability of plantlets throughout the year. *In vitro* propagation of pomegranate *Punica granatum* has been reported by several workers using different explant shoot tip and nodal explant (Murkute *et al.*, 2004, Singh and Khawale 2006, Chaugule *et al.*, 2007, Samir *et al.*, 2009, Singh *et al.*, 2011. However there is no report on callus and root induction through the mediation of *A. rhizogenes* without hormones in pomegranate. The objective of this study was to determine the effect of *A. rhizogenes* on callus of pomegranate.

Material and Methods:

Isolation of *Agrobacterium rhizogenes*: The strain of *Agrobacterium rhizogenes* was isolated from nodules of pea. The different culture media used for isolation were Yeast Mannitol broth; Luria Burtani (LB), Nutrient broth (NB) and Yeast extract peptone broth.

Confirmation of *Agrobacterium rhizogenes*: The *Agrobacterium rhizogenes* strain was designated as strain RPB13.

Pomegranate explant: *Invitro* micropropogated Pomegranate plants of variety Bhagwa, was selected for induction of callus and roots. The juvenile segments were multiplied on Murashige and Skoog medium (1962), supplemented with cytokinines. Culture conditions were 21±2ºC, photoperiod of 16 hrs. with a light intensity of 37 $\mu molm^{-2} s^{-1}$.

Bacterial culture for explant treatment: 48 hrs old cultures of *A. rhizogenes* strain RPB13 were taken and suspended in required quantity of sterile water to obtain 0.1 OD (read at 620 nm). This culture was used for treatment of micropropagated explants.

Callus induction: The juvenile segments were washed with detergent tween 20 for 4-5 min and then sterilized with 0.1 % $HgCl_2$ for 3-4 min and washed thoroughly with sterilized water to remove sterilants. For callus induction the explants were treated with *A. rhizogenes* and cultured on Murashige and Skoog (MS) medium without hormone. The data was recorded on % explant producing callus after 30 days.

Infection to Callus: The callus was punctured with hypodermic needles attached to a syringe containing an overnight culture of bacteria. The callus were co-cultivated with overnight bacterial cultures and incubated at 24ºC for two days in dark. Then, the callus was transferred to fresh MS medium without hormones. Successive transfers were made to make the incubating explants to free from bacteria and incubated under fluorescent light for root induction. The excess of bacterial cells present in the roots was eliminated by continuous sub culturing. The data was recorded on % of callus producing roots was recorded after 30-35 days.

Results:

Performance of *A. rhizogenes* for callus induction:

Pomegranate callus induction and survival of explants of pomegranate in control treatment (without hormone) was zero percent, indicating that hormones or *A. rhizogenes* are required for callus induction and survival for the explant under tissue culture condition (Table 1). Callus initiation was carried out by using juvenile segment as source of explant treated with *A.rhizogenes*. The explant showed swelling within 15-16 days of inoculation of *A.rhizogenes;* however callus formation started after 20-25 days at the margins of the explant and subsequently spread over the segment. The survivals of callus in the MS media having hormones were ranged from 80-85%. On an average 82% explants survived in the presence of hormonal treatment. However the survival of explant in *Agrobacterium rhizogenes* strain RPB13 treatment was 90%.

Performance of *A. rhizogenes* for root induction and root density. The induction of rooting by *A. rhizogenes* required fewer periods than that of hormonal treatment. *A. rhizogenes* treated callus required 35-40 days for root induction, whereas in hormonal treated explants required 55-60 days for root induction (Table 2). It indicated that the bacterium *A. rhizogenes* was more effective for induction of rooting and time required for root induction was 1-3 week less as compared to the hormonal treatment. Further the no. of roots after six week in pomegranate callus treated with *A. rhizogenes* was more as compared to hormonal treatment. The no. of roots formed with hormone treatment was 7 whereas it was more than double in *A. rhizogenes* treatment. The percent increase in root numbers in pomegranate callus due to *A. rhizogenes* was 128.5% more over hormonal treatment. These result clearly indicated that the treatment of callus with *A. rhizogenes* was more effective than hormone treatment for induction of root. The rooting response of callus for root induction through *A. rhizogenes* (a) and with hormone (b) is positive (Fig1).

Since, the rooting hormones are very costly, this can be replaced by the rooting bacterium *A. rhizogenes* under tissue culture experiment for rooting purpose. It was also interesting to note that percentage of callus induction and root generation due to *A. rhizogenes* were more as compared to hormone treated.

Table 1. *Invitro* performance of *Agrobacterium rhizogenes* on juvenile segments of pomegranate.

Explants	Treatment	No. of explants*	Survival (%) in plant	% of callus developed
Juvenile segments	*A. rhizogenes*	5	100	90%
	With Hormone	5	80	82%
	Control (without Hormone)	5	0	-

(+) - Induction of roots * Infection was done in Petriplates

(-) - No root induction

Table 2. Performance of *A. rhizogenes* on Pomegranate callus for root induction.

Explants	Treatment	Days for induction of roots	No. of roots after six weeks of infection	% increase in root no. over hormone Treatment
Callus	*A. rhizogenes*	35-40	16	128.5
	With Hormone	55-60	7	-

(a) **(b)**

Fig 1: Rooting response of Pomegranate callus for root induction through *A. rhizogenes* (a) and with hormone (b).

Discussion:

The results obtained during the present investigation indicated that the use of *A. rhizogenes* strain can be a successful approach for callus and root induction in pomegranate. For instance, the callus and root induction was poorest in the pomegranate explants without *A. rhizogenes* treatment. However, when this callus was inoculated with *A. rhizogenes* strain RPB13 without application of auxins cultures in ½ MS rooting medium, these significantly had higher number of roots as compared to control. Thus exogenous auxin was not required for the *A. rhizogenes* strain to induce higher root number and root length. Patena *et al* ., (1997 and 1998) also reported that in vitro shoots of "Golden Delicious" apple (Malus sylvestris) which had not been able to root in culture for 2 years when were treated with 4 strains of *A. rhizogenes* without auxin, two strains, A4 and 232 successfully induced rooting on these shoots. It is possible that micro- cuttings inoculated with the bacterium produced auxin since *A. rhizogenes* is known to encode genes that increase auxin sensitivity to the plant tissue (McAfee *et al.,* 1993; Hatta *et al* . 1996). In this study the best rooting response was obtained when the callus was inoculated with *A. rhizogenes* strain before they cultured in semi-solid ½ MS medium supplemented with 30 g/l sucrose without any hormone application. Monticelli *et al.,* 1997 also found that Almond and Ferragens with different rooting ability when infected in vitro with *Agrobacterium rhizogenes* 1855 induced root formation. In Ferragnes, root induction was strongly increased (55.6 %) with *A. rhizogenes* by infection without plant growth hormones in comparison to control (6.9 %) and the root induction was probably due to an improvement of rooting environment by the bacterium (McAfee *et al*., 1993; Simpson *et al.,* 1986; Li and Leung 2003). Giri *et al*., 2001, also stated that *A. rhizogenes* can be used for the efficient rooting of the cutting from recalcitrant woody species, as under highly defined experimental conditions, the process of root formation can be easily manipulated and the rooting response could be relatively high. Thus, this approach is attractive for the mechanism of root induction from callus by *A. rhizogenes* in pomegranate. This *A. rhizogenes* strains significantly developed callus and root number. Thus the application of *A. rhizogenes* appears to be very useful for micropropagation of Pomegranate that are difficult to root.

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