Effective Induction of Hairy Roots in Persian Poppy (*Papaver bracteatum* Lindl.) Using Sonication Method

Qavami N (Ph.D. Student)[1], Azizi M (Ph.D.)[1]*, Yazdian F (Ph.D.)[2], Qaderi A (Ph.D.)[3], Nemati H (Ph.D.)[1]

1- Department of Horticultural Sciences, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
2- Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran
3- Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran
* Corresponding author: Ferdowsi University of Mashhad, Department of Horticultural Science, Mashhad, Iran
Tel: +98- 51-38805753
E-mail: azizi@um.ac.ir

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Abstract

Background: ***Papaver bracteatum*** **Lindl. Commonly known as Iranian poppy is an important medicinal plant due to the presence of benzylisoquinoline alkaloids.**

Objective: To evaluate the hairy root culture as a novel method for thebaine production.

Methods: To optimize the hairy root culture of *P. bracteatum*, five strains of *Agrobacterium rhizogenes* (ATCC15834, C318, A13, 9453 and A4) were used by means of Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT).

Results: Strain C318 proved to be more efficient than the other strains to hairy root induction ability. Among different experimental conditions, the highest transformation rate (28.5 %) was observed 4 weeks after inoculation, once the explants were subjected to sonication (45 s) following a heat treatment of 41°C for 5 min. Transgenic status of the hairy roots was confirmed by PCR using *rolB* specific primers.

Conclusion: Strain C318 proved to be more efficient than the other strains to hairy root induction ability. Among different experimental conditions, the highest transformation rate (28.5 %) was observed 4 weeks after inoculation, once the explants were subjected to sonication (45 s) following a heat treatment of 41°C for 5 min. Transgenic status of the hairy roots was confirmed by PCR using *rolB* specific primers.

Keywords: ***Papaver bracteatum*** **Lindl. Hairy root, Sonication-assisted *Agrobacterium*-mediated transformation, Thebaine**

Introduction

Iranian poppy (*Papaver bracteatum* Lindl.) is an important medicinal plant because of its benzylisoquinoline alkaloids [1]. The plant is naturally distributed in the mountains range of Alborze and Zagros, Iran. The main alkaloid of Iranian poppy (*P. bracteatum*) is thebaine, which is used for semisynthetic codeine production [2-4]. In recent years the global demand of thebaine has increased due to its non-narcotic properties and being an important component for the manufacture of a number of opiates as oxycodone, oxymorphine, buprenorphine, etc. This plant can be a potential alternative to *Papaver somniferum* for production of codeine. However, the perennial nature of *P. bracteatum* and problems related to field grown plants makes it uneconomical for this replacement [5, 6]. To overcome these problems and also to shorten the time of production, advanced biotechnological methods like hairy root culture could be used. The cultivation of hairy roots is an in vitro method that has many advantages for commercial production. Hairy roots are developed by infecting explants with *Agrobacterium rhizogenes*, a gram-negative soil bacterium that integrates a DNA segment (T-DNA) into the host plant genome which results in the active proliferation of the roots [7, 8]. Compared to normal root cultures, hairy roots are fast-growing, genetically and biochemically stable, easy to maintain, and are able to grow in phyto-hormone free media. They offer a continuous source of secondary metabolites which are important for pharmaceuticals, cosmetics, and food additives. They are especially useful for the production of secondary metabolites that are synthesized in roots and then accumulated in aerial parts of the plant [9, 10]. Hairy root induction is done in a variety of ways. Technique named sonication-assisted *Agrobacterium* –mediated transformation (SAAT) has been developed to induce hairy roots in those plant species which are difficult to transform. Trick and Finer (1997) observed that SAAT treatment produces small, uniform fissures and channels in tissues of various plants which facilitate the access of *A. rhizogenes* to the internal plant tissues [11]. This technique involves subjecting the plant tissue to brief periods of ultrasound in the presence of agrobacterium [12]. SAAT successfully has been applied for hairy root induction in *P. somniferum* [13] and *Verbascum xanthophoeniceum* [14]. The present study was conducted with the major objective of improving the transformation efficiency of *A. rhizogenes* for hairy root induction in *P. bracteatum* by sonication devise. This system provides an efficient tool for getting better transformation and is useful for commercial *in vitro* production of benzylisoquinoline alkaloids.

Material and methods

Seed sterilization

The mature seeds of *P. bracteatum* were collected from Anjomaneh area in Kurdistan Province, Iran. The seeds were surface sterilized with 70 % (v/v) ethanol for 1 minute and 2% (v/v) sodium hypochlorite solution for 15 min. Then, seeds were rinsed five times with sterilized water. The basal medium containing 1/2 MS [15] salts and vitamins, was solidified

with 0.6 % (w/v) agar. The medium was adjusted to pH 5.8 before adding agar and was sterilized by autoclaving at 121°C for 20 min. The seeds were germinated at 22°C under a 16/8 h (day/night) photoperiod in phytotron.

Sonication and heat treatment on hairy root induction

A. rhizogenes strains (9435, A4, A13, C318 and MTCC 15834) were provided by Institute of Medicinal Plants (IMP), and stored at -80°C. The bacteria were grown overnight in liquid LB (Luria-Bertani) medium supplemented with 50 mg/l rifampicin, OD_{600nm} = 0.6-0.8 at 28°C on a shaker incubator (100 rpm) in dark. The bacterial suspensions were centrifuged at 4000 rpm for 10 min and resuspended in liquid inoculation medium, which was MS medium containing 50 mg/l sucrose [4], and 100 µM acetosyringone.

For the SAAT experiments explants including leaves, hypocotyls and excised shoots of 1-month-old papaver were cut and transferred to the sterile 50 ml falcon tubes containing inoculation medium (as explained above). The plastic tubes were individually placed in a sonicator and subjected to an ultrasound with frequency of 40 KHz (ultrasonic cleaner bath; Pars Nahand, Tehran). The duration of treatments considered as 15, 30, 45 or 60s followed by heat treatment at 41°C for 5 min.. Thereafter, explants were blotted on filter paper (Whatman No.1) to remove excess bacteria and then transferred to co-cultivation medium (MS) in dark. After 2 days co-cultivation, all the explants were transferred to the solid MS media, supplemented with 30 g/l sucrose, 300 mg/l cefotaxime sodium. Hairy roots emerged from the wound sites of the explants within 3-4 weeks. The hairy roots were excised from the explant tissues and sub-cultured on hormone free MS medium at 25±2°C in the dark every 2 weeks. The concentration of cefotaxime was gradually decreased and finally was omitted after 2 months of culture [4].

Hairy root induction was confirmed by Polymerase Chain Reaction (PCR) test using *rolB* and *vir*D specific primers (Table 1). The total genomic DNA from hairy root and natural root were isolated according to the CTAB (cetyl trimethylammonium bromide) method [16]. Also the plasmid DNA from *A. rhizogenese* was prepared by alkaline lysis with SDS [17]. Polymerase chain reaction (PCR) was performed according to Grabkowska et al. with the following modification [**18**].

Establishment of hairy roots and HPLC analysis

Three to four weeks old hairy roots were subcultured into a hormone-free MS liquid medium. The cultures were maintained at 90 rpm in an orbital shaker under 25 ± 2°C in 24 h darkness. Hairy roots were subcultured to fresh media every 15 days of culture. HPLC analysis of thebaine was performed according to modified method base on BP 2013 [19]. The conditions of the HPLC set are presented in Table 2.

Table 1- The sequences of *rolB* and *virD* primers and Thermal Cycler Condition of Polymerase Chain Reaction

Genes	Primer Sequences	Denaturation Temp.(°C)	Annealing Temp. (°C)	Extension Temp. (°C)	Number of cycle	Amplified segment (bp)
rolB	Forward:5'-GCTCTTGCAGTGCTAGATTT-3' Reverse:5'- GAAGGTGCAAGCTACCTCTC-3'	95	55	72	35	423
VirD	Forward: 5'-GCTCTTGCAGTGCTAGATTT-3' Reverse: 5'-GAAGGTGCAAGCTACCTCTC-3'	95	59	72	35	438

Table 2- Chromatography set conditions

Detector	UV
Column	CN/4.9×250 mm
Flow rate	1.5 ml/min
Detector wavelength	254 nm
Standards concentration	100, 250, 500, 1000 ppm
Mobile phase	Mixing of Ammonium acetate, Dioxane, Acetonitrile & Methanol
Inject volume	20 µl
Apparatus	Knauer Pump K-1001 Detector K-2501
Temperature	Room temperature
Retention time	About 10 min.

Statistical analysis

The transformation experiments were set up in a factorial arrangement based on completely randomized design with three replications. All data were subjected to one-way ANOVA, followed by the statistical significance test. The significant difference among the mean ± standard error (SE) was carried out using Duncan's Multiple range test and significance level of $P \leq 0.01$ (IBM, SPSS ver. 19).

Result and Discussion

Effect of *A. rhizogenes* strains and explant type on hairy root induction

The results showed that all *A. rhizogenes* strains ((C318, 9534, A13, A4 and MTCC 15834) had the ability of induction of hairy roots in *P. bracteatum* explants (Fig. 1). Transformation rate and frequency of hairy roots induction significantly depended on the applied *A. rhizogenes* strains and explants types. The highest rate of hairy root induction was obtained for excised shoot explants by C318 strain (28.5%), while A13 strain induced hairy roots in only 9% of the hypocotyl explants. The hypocotyl explants showed necrosis with a low rate of hairy root induction (Fig. 2). The difference in the induction of hairy roots between strains could be due to the difference in virulence of various strains of *A. rhizogenes*. Sharafi *et al.* showed successfully using of excised shoot explants for transformation of Iranian poppy [4]. Rate of hairy roots induction was found to be low in the leaf and hypocotyl explants (Fig. 2). Influence of *A. rhizogenes* strain and the type of explants on hairy root induction has been documented earlier in several plant species [20, 21].

Fig. 1- *A. rhizogenes* mediated transformation of *P. bracteatum*. (a) hairy root induction in leaf explants; (b) induction of hairy root on wound site of excised shoot

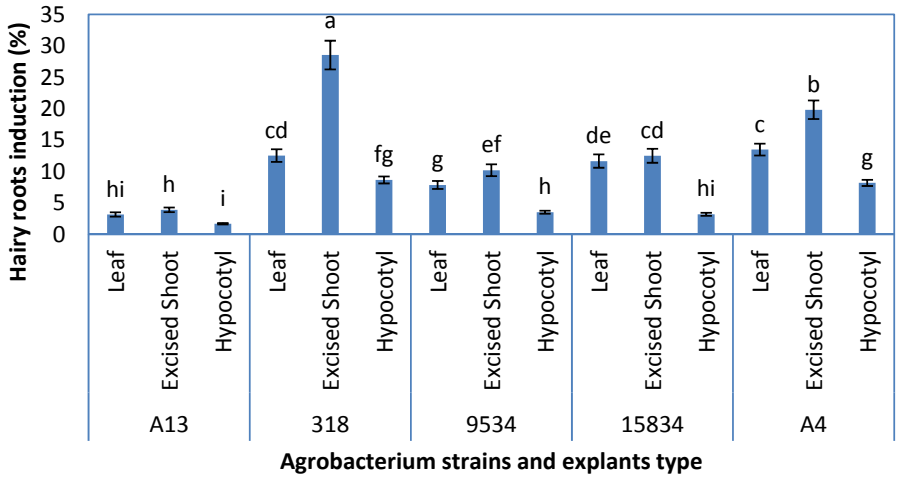


Fig. 2- Effect of different bacterial strains and explants on percentage of hairy root induction of *P. bracteatum*. Data are provided as a mean of three replications. Vertical lines represent standard error

Effect of sonication on hairy root induction

Sonication-assisted *Agrobacterium*-mediated genetic transformation (SAAT) is an improved approach to induce hairy roots formation in plant species, which are difficult to transform [13]. The enhanced transformation efficiency using SAAT, probably results from thousands of microwounds on and below the surface of the target tissue stimulated by sonication. These microwounds facilitate Agrobacterium penetration into the target tissue and provide efficient delivery of T-DNA for transformation [11, 14]. We investigated the influence of sonication on transformation efficiency of *A. rhizogenes* in Iranin poppy by sonication of explants in suspension for time duration of 15, 30, 45 and 60s, which was followed by a heat treatment of 40°C for 5 min. Result showed that interaction sonication time with explants type and agrobacterium strains were significant ($P \leq 0.01$). The highest rate of hairy root induction was obtained for excised shoot explants (19.1%) and C318 strain (22%) in 45s sonication (Fig. 3-4). After 45s sonication, viability of types of explants decreased. Georgiev [22] reported the highest

percentage of hairy root induction (75%) was obtained through 45s ultrasound exposure. Le Flem-Bonhomme *et al*. [1] showed which this technique is effective for transforming the hypocotyl of *P. somniferum* plants but, there are no reliable report on SAAT method for hairy root induction in *P. bracteatum*. In all cases hairy roots appeared 3-4 weeks after transformation. It should be noted that the time for hairy roots formation depends on species, age and type of plant tissue. For instance, when Papaver hypocotyls were infected with *A. rhizogenes* LBA 9402 via ultrasound, hairy roots occurred after 5 weeks of culture [1, 22].

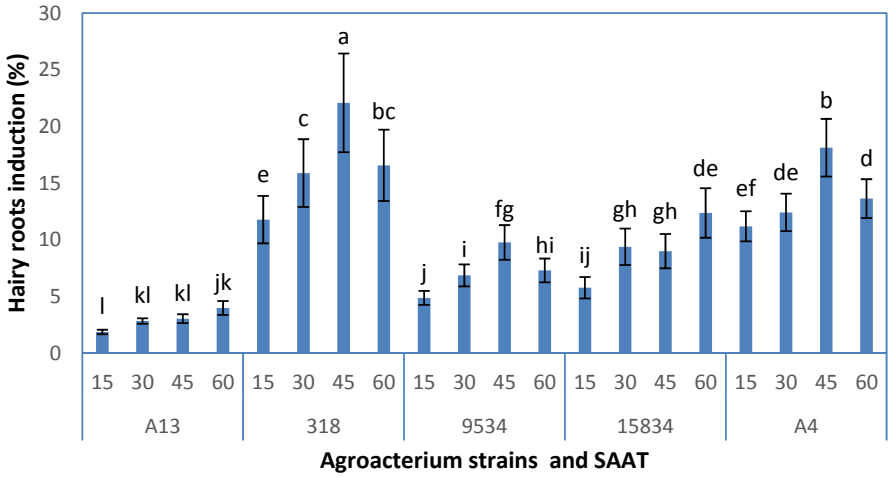


Fig. 3- Influence of sonication and Agrobacterium strains on transformation efficiency and hairy root induction. Data represent mean ± SE, of the three independent experiments.

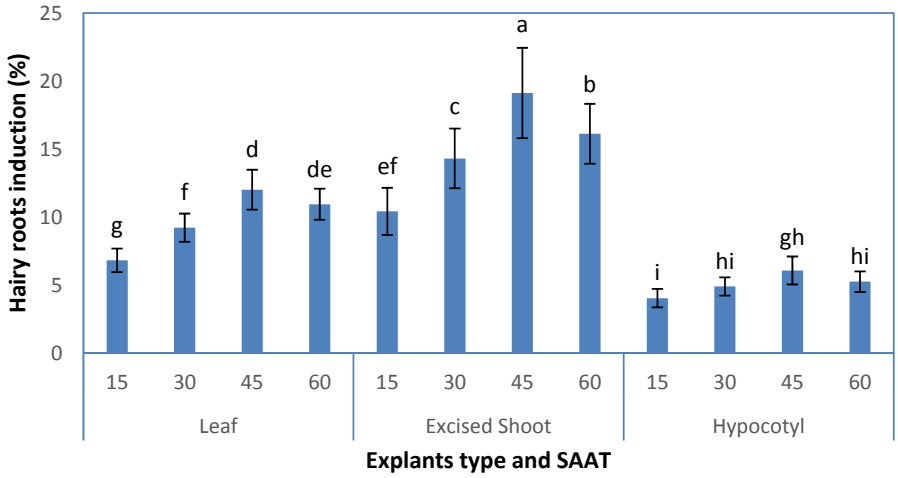


Fig. 4- Influence of sonication and explants type on transformation efficiency and hairy root induction. Data represent mean ± SE, of the three independent experiments

To confirm hairy roots induction, the insertion of T-DNA segments in root genome was verified by PCR analysis with specific genes primers of *rol*B (Fig. 5).

Establishment of hairy root culture and HPLC analysis

The potential growth of hairy roots in liquid (MS) shake flask culture without hormone was successfully achieved in the present study. The HPLC profile of thebaine in induced hairy root is shown in Fig. 6. Data confirmed a considerable amount of thebaine (4.34 mg/g), which was identified with peaks corresponding to standard thebaine markers.

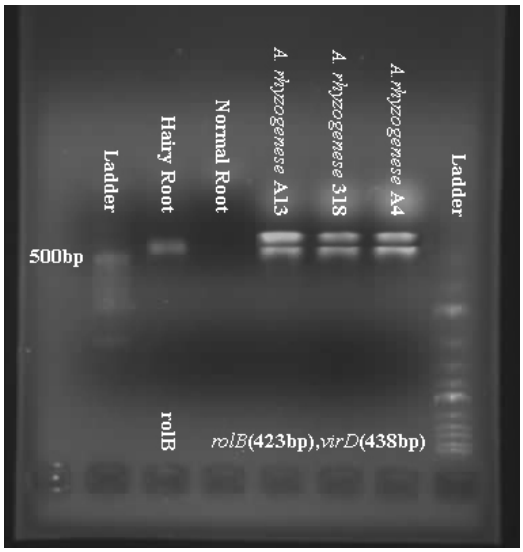
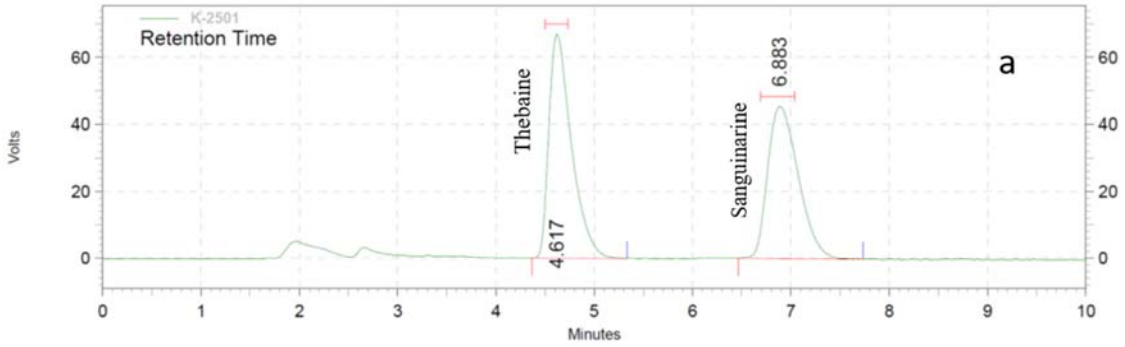


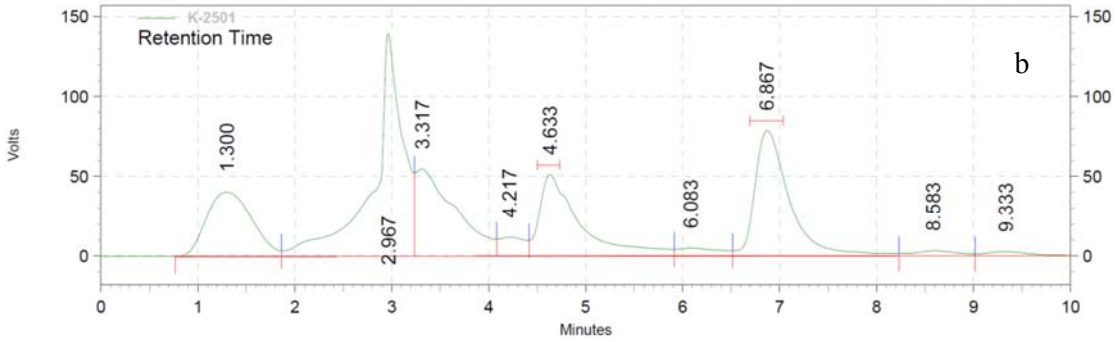
Fig. 5- PCR detection of *rol B* gene in hairy roots



K-2501 Results

Retention Time	Area	Area %	Height	Height %
4.617	1071173	52.05	67027	59.62
6.883	986830	47.95	45400	40.38

Fig. 6-



K-2501 Results

Retention Time	Area	Area %	Height	Height %
1.300	1311072	13.45	40525	10.47
2.967	2925581	30.02	139655	36.09
3.317	1566436	16.07	54786	14.16
4.217	219025	2.25	11903	3.08
4.633	1483564	15.22	51185	13.23
6.083	146499	1.50	4884	1.26
6.867	1935793	19.86	78651	20.33

Fig. 6 - HPLC analysis of Thebaine production in hairy root culture. a: Standard Thebaine, b: Thebaine from hairy roots

The accumulation of higher levels of thebaine in hairy roots could be explained due to the effect of T-DNA integration on the secondary metabolite production as reported in many plant species [20]. In accordance with our findings, Sharafi *et al.* [4] and Rostampour *et al.* also confirmed the presence of benzylisoquinoline alkaloids in transformed hairy root cultures of *P. bracteatum.*

Conclusion

The highest percentage of hairy root induction was obtained with *A. rhizogenes* strain C318 in *P. bracteatum* excised shoot explants through 45s sonication. In the other conditions also hairy root growth was observed; however, the transformation frequency was significantly lower (Fig. 3-4) and even necrosis of explants was observed which exposed to 60s ultrasound. These transformed hairy roots are able to produce bioactive compounds. To the best of our knowledge, this is the first report of *A. rhizogenes*-mediated transformation using sonication method in *P. bracteatum*. This system provides an efficient tool for hairy root production and seems beneficial for commercial production of thebaine under *in vitro* condition.

References

1. Le Flem-Bonhomme V, Laurain-Mattar D and Fliniaux M. Hairy root induction of *Papaver somniferum* var. album, a difficult to transform plant, by *A. rhizogenes* LBA 9402. *Planta Medica*, 2004. 218 (5): 890-893.

2. Milo J, Levy A and Palevi M, An Alternative Raw—The Cultivation and Breeding of Papaver Bracteatum, in Poppy, B. J, Editor. 1998, harwood academic: Singapore. pp: 279-289.

3. Rostampour S, Hashemi Sohi H and Dehestani A. In vitro regeneration of Persian poppy (*Papaver bracteatum*). *Biologia* 2010; 65 (4): 647 - 652.

4. Sharafi A, Hashemi Sohi H, Mousavi A, Azadi P, Razavi K and Otang Ntui V. A reliable and efficient protocol for inducing hairy roots in *Papaver bracteatum. Plant Cell Tissue Organ Culture* 2013; 113 (1): 1-9.

5. Chaterjeea A, Shuklaa S, Mishrab P, Rastogia A and Singh S. Prospects of in vitro production of thebaine in opium poppy (*Papaver somniferum* L.). *Industrial Crops and Products* 2010; 32: 668-670.

6. Park S and Facchini P J. Agrobacterium rhizogenes-mediated transformation of *Opium poppy*, *Papaver somniferum* L., and *California poppy*, Eschscholzia California Cham., root cultures. *J. Experimental Botany* 2000; 51: 1005-1016.

7. Srivastava S and Srivastava A. Hairy root culture for mass-production of high-value secondary metabolites. *Biotechnology Inform Healthcare* 2007; 27 (1): 29-43.

8. Gaia Q, Jiao J, Luoa M, Wang W, Ma W, Zu Y and Y F. Establishment of high-productive *Isatis tinctoria* L. hairy root cultures: A promising approach for efficient production of bioactive alkaloids. *Biochemical Engineering J*. 2015; 95: 37 - 47 Contents.

9. Zhou M, Zhu X, Shao J, Tang Y and Wu Y. Production and metabolic engineering of bioactive substances in plant hairy root culture. *Applied Biochemistry and Biotechnol.* 2011; 90: 1229 - 1239.

10. Georgiev M, Pavlov A I and Bley T. Hairy root type plant in vitro systems as sources of bioactive substances. *Applied Biochemistry and Biotechnol.* 2007; 74: 1175 - 1185.

11. Trick H and Finer J. SAAT: Sonication-assisted Agrobacterium -mediated transformation. *Transgenic Res.* 1997; 6: 329 -336.

12. Beranova M, Rakousky S, Vavroa Z and Skalicky T. Sonication assisted Agrobacterium-mediated transformation enhances the transformation efficiency in flax (*Linum usitatissimum* L.). *Plant Cell, Tissue and Organ Culture* 2008; 94 (3): 253-259.

13. Subramanyam K, Sailaja K, Srinivasulu M and Lakshmidevi K. Highly efficient Agrobacterium-mediated transformation of banana cv. Rasthali (AAB) via sonication and vacuum infiltration. *Plant Cell Reports* 2011; 30: 425-436.

14. Thilip C, Raju C, Varutharaju K, Aslam A and Shajahan A. Improved Agrobacterium rhizogenes-mediated hairy root culture system of *Withania somnifera* (L.) Dunal using sonication and heat treatment. *Biotechnol.* 2015; 5 (6): 949-956.

15. Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiology Plant* 1962; 15: 473-497.

16. Doyle J and Doyle J. Isolation of plant DNA from fresh tissue. *Focus* 1990; 12: 13-15.
17. Sambrook J and Russell D. Molecular Cloning: A Laboratory Manual. 3 ed. Vol. 3. 2001, New York: Cold Spring Harbor Laboratory. 2100.
18. Grabkowska R, Krolicka A, Mielicki W, Wielanek M and Wysokinska H. Genetic transformation of Harpagophytum procumbens by *Agrobacterium rhizogenes*: iridoid and phenylethanoid gly- coside accumulation in hairy root cultures. *Acta Physiology Plant*. 2010; 32: 665 - 673.
19. U K S O. British Pharmacopoeia 2013. 2012, U.K: Stationery Office.
20. Khatodia S and Biswas K. A comparative study of Hairy Root Culture induction efficiency in four medicinally important plants using *Agrobacterium rhizogenes. International Journal of Current Microbiology and Applied Science* 2014; 3 (5): 625-633.
21. Zhou M, Zhu X, Shao J, Wu Y and Tang Y. An Protocol for Genetic Transformation of *Catharanthus roseus* by *Agrobacterium rhizogenes* A4. *Applied Biochemistry and Biotechnol.* 2012; 166: 1674 - 1684.
22. Georgiev M, Ludwig-Muller J, Alipieva K and Lippert A. Sonication-assisted Agrobacterium rhizogenes-mediated trans- formation of *Verbascum xanthophoeniceum* Griseb. for bioactive metabolite accumulation. *Plant Cell Reports* 2011; 30: 859-866.