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Research Article

Optimization of factors affecting *Agrobacterium*-mediated hairy root induction in *Vitex negundo* L. (Lamiaceae)

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Abstract: *Vitex negundo* L. is an aromatic, woody, blooming shrub in the Lamiaceae family which can grow into a small tree. Traditionally, *V. negundo* root has been used to cure diabetes, colic, boils, leprosy, and rheumatism. Keeping the importance of its roots in mind, an attempt has been taken for development of a protocol for efficient hairy root proliferation system. The *Agrobacterium rhizogenes* strains (MTCC 532 and MTCC 2364) were used for hairy root induction. For *A. rhizogenes* infection, both *in vitro* and *in vivo* leaves as well as internodes were used as explant. *In vitro* leaves and internodal explants were obtained by the inoculation of matured nodal segments on the optimum medium [MS + 2.0 mg/L N^6-Benzylaminopurine (BAP)] with *c.a.* 91.6% shoot regeneration and an average of 8.1 shoots per explants. *In vitro* leaf showed best hairy root induction followed by *in vitro* internode on ½ MS medium augmented with acetosyringone. Highest transformation efficiency was achieved using MTCC 2364 strain, while no transformation was observed in MTCC 532 strain. Different factors affecting transformation including co-cultivation period, infection time and optical density (O.D.) value were standardized. The highest efficacy, 88.8% hairy root induction was observed in *in vitro* leaves infected by MTCC 2364 for 60 minutes infection time with an O.D. value of 0.29 maintained over a 44-48 hours of co-cultivation period. The prescribed protocol may be used as a reference for development of industrial scale hairy root production for bioactive compound located in root of *V. negundo*.

1. INTRODUCTION

Vitex negundo L. (Lamiaceae) is an aromatic, woody and blooming shrub found mainly in India, Ceylon and China. The plant is also found at an altitude of 1500 m in the outer Himalayas (Usha *et al*., 2007). The term "*Vitex*" is originated from the Latin word 'vieo', meaning "to weave together", because of the flexible quality of the twigs and stems of the plant (Ahuja *et al*., 2015). *V. negundo* is usually an agroforestry plant and also used as fence. Besides, the plant is a traditional medicinal plant that has also been adopted in modern medicine. From root to fruit,

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the plant contains a variety of secondary metabolites that enhance the plant's ability to provide matchless medical benefits (Singh *et al*., 2016). Leaf is aromatic, used as tonic, and a vermifuge. The secondary metabolites present in the root juice (i.e. Dibutyl malate, Ajmalicine, Bruceantin, Beta carotene, Stearoyldelicone etc.) are expectorant, used as tonic for diuretic property and also used to treat colic, worms, rheumatism, dyspepsia, leprosy and boils (Gade *et al*., 2023; Ghani *et al*., 1998). Fruit is helpful for cerebrospinal, anxiety and emmenagogue therapies; dried fruit is a vermifuge, and blossoms are employed in cold and astringent treatments (Nadkarni *et al*., 2002). Scrofulous ulcers and sinuses are treated with floral oil of this plant. *V. negundo* has also been found as an antimicrobial (Dewade *et al*., 2010) and anti-inflammatory drugs (Chawla *et al*., 1992). Powdered seeds are used to cure spermatorrhoea and can be utilized as an aphrodisiac when mixed with milk and dried *Zingiber officinale* (Yunos *et al*., 2005).

Due to its immense medicinal property the plant plays an important role in pharmaceutical industry. Further secondary metabolite profiling of the *V. negundo* roots depicted about the presence of vitexin, isovitexin, negundin-A, negundin-B, acetyl oleanolic acid, sitosterol, alkaloids, phenols, flavonoids, glycosidic irridoids, tannins and terpene (Ladda & Magdum, 2012). Biotechnological intervention is useful for development of various efficient strategies to explore the therapeutic potential of *V. negundo.* Production of aerial parts like leaf, fruit, seed and flower has been already achieved by a number of workers (Vishwanathan & Basavaraju, 2010; Rana & Rana, 2014; Kamal *et al*., 2022) through *in vitro* regeneration of shoot using different explants like node and internode. But for large scale *in vitro* root production in *V. negundo* has not yet been reported in spite of being the source of most of the active compounds of pharmaceutical importance. *A. rhizogenes* interacts with plant cells through a defined segment of its large root inducing plasmid DNA (pRi T-DNA), which is conjugationally transferred into the plant genome and expressed. This results in the development of a tumors "hairy root" disease, distinguished by localized adventitious root proliferation at the infection site, and hiding two auxin biosynthetic genes (iaaM, iaaH). Under *in-vitro* culture, these genetically modified roots exhibit a rapid rate of growth along with abundant lateral branching, negative geotropism, cytogenetic stability, and sustained growth and biosynthetic potential in the medium without the need for growth regulator input (Sahu *et al*., 2015). In this context, *in vitro* root cultures in particular hairy root production could be the most appropriate alternative method which will be useful for secondary metabolites production in order to fulfill the requirement of drug designing and manufacturing. The advantages of production of secondary metabolites through hairy root culture also includefast growth, low doubling time, ease of maintenance of hairy roots, and their ability to synthesize a wide range of chemical compounds which could be a continuous source for the production of valuable secondary metabolites. To successfully induce and establish hairy root cultures of *V. negundo* using *A. rhizogenes*, various factors impacting genetic transformation must be meticulously evaluated and standardized. These include explant source and type, culture matrix, bacterial strains, bacterial cell density (by O.D. “Optical Density”), acetosyringone pre-culture, method of infection, infection time and duration of co-cultivation. Therefore, for the first time, efforts were made to optimize different factors to maximize the efficiency of the transformation and to develop a reproducible protocol for hairy root production in *V. negundo* which could serve as a feasible and sustainable source of pharmaceutically important phytochemicals.

2. MATERIAL and METHODS

2.1. *In vitro* Shoot Proliferation for *Agrobacterium rhizogenes* Infection

The *Vitex negundo* species was collected from Athagarh, Cuttack, Odisha, India and established in the experimental garden of Department of Botany, Ravenshaw University, Cuttack, Odisha (Figure 1 a). The *V. negundo* nodal explants were surface sterilized by washing with running tap water for 30 minutes, then treating with 2% Teepol (Reckitt Benckiser Ltd., India) for 10

minutes followed by 2% Bavistin (Antracol fungicide, Bayer, Crystal Crop Protection Ltd., India) treatment for 10 minutes. Finally, the explants were treated with 0.1% $HgCl_2$ (Merck, India) for 7 minutes as the last step of surface sterilization. For multiple shoot proliferation, the nodal explants were inoculated on different strengths of Murashige and Skoog's (1962) (MS) medium i.e., MS, ½ MS, ¼ MS, ⅛ MS either alone or, MS medium augmented with various concentrations (0.5 to 3.0 mg/L) of Benzylaminopurine (BAP; Hi Media, India). The well-developed *in vitro* shoots (Figure 1 b) were served as a source of *in vitro* explants (Leaf and Internode) for *A. rhizogenes* infection.

2.2. Maintenance of Bacterial Culture

The *A. rhizogenes* cultures MTCC 532 and MTCC 2364 (CSIR-IMTECH, MTCC, Chandigarh) were revived in nutrient broth (1.3 g/100 mL; Hi media, India) with a pH of 7.4±0.2. For bacterial growth, the temperature was set at 26±1°C for 24 to 48 hours inside incubator (Shaker and Incubator, N. Biotek, NB 205 QF). The O.D (Optical Density) of both bacterial cultures, MTCC 2364 and MTCC 532, maintained in between 0.02 and 0.8. after 24 to 48 hours of incubation were used for hairy root induction. After revival of both the strain in nutrient broth, the cultures were stored and maintained on Nutrient agar medium (1.3 g/100 mL nutrient broth and 1.8 g/100 mL bacteriological grade agar (Himedia, India) inside a refrigerator at 4-5°C for future use (Figure 1 e and f).

2.3. *Agrobacterium* Infection and Hairy Root Induction

2.3.1. *Transformation and hairy root induction in internodes*

For transformation and hairy root induction experiment, both *in vitro* and *in vivo* internodes were used. *In vitro* shoots from a 45 to 60-days-old culture were cut into small pieces (1.0 - 2.0 cm) and the excised *in vitro* internodes were punctured at one end. The punctured side was dipped in the bacterial suspension for different infection time 15-75 minutes. After the infection, the internodes were soaked with sterile tissue paper and inoculated on ½ MS and half MS + acetosyringone (19.6 mg/mL; Hi-Media; Figure 1 c) media by the other side of the piercing. These ½ MS media were gelled with 0.6% agar and pH was adjusted to 5.8±0.01. The co-cultivation time was optimized as 44-48 hours inside an incubator at 26°C. After 44-48 hours of co-cultivation, the internodes were transferred into a new flask with ½ MS + acetosyringone medium and kept in a culture room at 24°C under dark condition for hairy root induction. *Agrobacterium* infection in the *in vivo* internode was carried out, after surface sterilization, in the same way as *in vitro* internodes. The procedure of surface sterilization of the *in vivo* internode explant was carried out following the same procedure mentioned earlier for mature nodal explants.

2.3.2. *Transformation and hairy root induction in leaves*

Forty-five to sixty days old axenic shoot cultures were taken as the source for *in vitro* leaves. The leaves were cut into small segments (0.5 cm width ×1 cm length) and a sterile needle was used to pierce the mid rib and entire surface of the leaves. The pierced leaves were dipped in the bacterial suspension for 15-75 minutes. The leaves were dried with sterile tissue paper before being inoculated on ½ MS medium (Hi-Media, India; pH 5.8±0.01) and ½ MS + acetosyringone (Stock :196 mg/ 10 mL i.e., 0.1 M, Hi-Media, India; pH 5.8±0.01; Figure 1 d) media gelled with 0.6% agar. The co-cultivation time was optimized between 44-48 hours at 26°C inside an incubator. After 44-48 hours of co-cultivation, the leaves were transferred to fresh flasks containing ½ MS + acetosyringone (0.1 M) medium and kept in dark inside a culture room at 24°C. The *in vivo* leaves, after following the surface sterilization procedure were subjected to *A. rhizogenes* infection in the same way as *in vitro* leaves.

2.4. Statistical Analysis

All values are presented as the mean with three biological replicates. Mean values within column with different superscript alphabets are significantly different. Data were analyzed by analysis of variance (ANOVA) using Duncan's multiple range test ($p < 0.05$).

3. FINDINGS

3.1. *In vitro* Shoot Proliferation from *in vivo* Nodal Explants

Out of different strengths of MS basal media tried for *in vitro* shoot multiplication from *in vivo* nodal explants, the full-strength MS was found better in terms of shoot proliferation percentage, shoot number and shoot length whereas all these parameters were enhanced after the addition of BAP in different concentrations into the full-strength MS. The shoot regeneration from the nodal segments was observed after 7 days of inoculation of explants. Highest percentage of shoot regeneration (91.6) with highest number (8.1) of shoots with 8.33 cm average shoot length were recorded on MS medium augmented with BAP (2.0 mg/L) after 40-50 days of inoculation (Table 1).

Table 1. *In vitro* shoot proliferation from *in vivo* nodal explant of *Vitex negundo.*

Sl. No.	Media	Percentage of shoot proliferation	Mean shoot number	Mean shoot length (in cm)
1	⅛ MS	50.0^{f}	1.0^{ef}	0.38^{efg}
2	¼ MS	50.0^{f}	1.3^{e}	0.41^{ef}
3	½ MS	58.3^{de}	1.3^{e}	0.63^{def}
4	MS	62.5^{d}	1.5^{de}	0.88^{de}
5	MS + BAP (0.5mg/L)	62.5^{d}	1.6^{d}	1.51^{d}
6	MS + BAP (1.0mg/L)	66.6^{cd}	2.3^{cd}	2.16^{cd}
7	MS + BAP (1.5mg/L)	70.8^{c}	2.5^{c}	3.16^{c}
8	MS + BAP (2.0mg/L)	91.6^{a}	8.1^{a}	8.33^{a}
9	MS + BAP (3.0mg/L)	79.1^{b}	3.4^{b}	7.13^{ab}

Data pooled from 02 explants per flask, 04 flasks per replication and the experiments were repeated 03 times (2×4×3 =24). Each value is the mean from 3 replications. Mean values showed by different letters are significantly different at $p < 0.05$ (DMRT).

3.2. Induction of Hairy Roots

For *Agrobacterium* transformation, we tested four different explant types, including *in vivo* (leaf and internode) and *in vitro* (leaf and internode). Further, two different media types, including ½ MS without acetosyringone and ½ MS with acetosyringone, were employed for the inoculation after *Agrobacterium* infection. There was no rooting tendency in the two sets of control group i.e., *in-vivo* and *in-vitro* (internodes and leaves) explants of ½ MS and ½ MS with BAP medium, which were not infected by *A. rhizogenes*. However, only the *in vitro* (leaf and internode) inoculated on the ½ MS with acetosyringone medium showed positive response for hairy root induction.

3.2.1. *Hairy root induction from internode*

The *A. rhizogenes* strains MTCC 2364 and MTCC 532 were subjected for hairy root induction from both *in vivo* (mature) internode and *in vitro* internode, among which *in vitro* internode showed hairy root induction with MTCC 2364 strain. Different infection time (15, 30, 45, 60 and 75 minutes), O.D. values (0.042, 0.375 and 0.572) with co-cultivation time (44-48 hours) were examined for hairy root induction and the results were depicted in Table 2. The initiation of hairy root was observed after 15 days of infection whereas on the 30th day of observation i.e., with 30 minutes infection time 2.27 root numbers (1.66 cm), with 45 minutes infection time 1.5

root numbers (1.25 cm), 0.66 root numbers (0.83 cm) root length and with 60 minutes infection time 2.90 root numbers (2.92 cm) were observed. The rest of the explants of *in vitro* internode did not show any hairy root induction rather eventually all these explants became brown and died after few days of infection (Table 2). Overall, at O.D. value 0.042 with 60 minutes infection time and 44-48 hours co-cultivation period exhibited best results for hairy root induction (Table 2; Figure 1 g). Another strain MTCC 532 was not found effective with any type of explants tested for hairy root induction. It is noteworthy to explain that during co-cultivation period the explant inoculated on ½ MS medium fortified with acetosyringone only got success in hairy root transformation but explant inoculated on ½ MS without any addition of acetosyringone failed to respond.

Table 2. Factors affect hairy root induction in *in vitro* internode infected by MTCC 2364.

O.D.	Infection Time (in minutes)	Co-cultivation Period (in hours)	Average Hairy root induced (%)	Number of induced Hairy root (average)	Length of Hairy root (average)
0.042	15	44-48	0	0	0
	30		0	0	0
	45		22.21^{bc}	1.5^{abc}	1.25^{bc}
	60		66.65^{a}	2.90^{a}	2.92^{a}
	75		0	0	0
0.375	15	44-48	0	0	0
	30		38.88^{b}	2.27^{ab}	1.66^{b}
	45		0	0	0
	60		0	0	0
	75		0	0	0
0.572	15	44-48	0	0	0
	30		0	0	0
	45		11.10^{d}	0.66^{bc}	0.83^{c}
	60		0	0	0
	75		0	0	0

Data pooled from 03 explants per flask, 02 flasks per replication and the experiment repeated 03 times (3*2*3=18). Each value is the mean from 3 replications. Mean values showed by different letters are significantly different at $p < 0.05$ (DMRT).

3.2.2. *Hairy root induction from leaves*

The *A. rhizogenes* strains MTCC 2364 and MTCC 532 were also used for infection of both matured *in vivo* leaves and *in vitro* leaves, among which *in vitro* leaves showed hairy root induction with MTCC 2364 strain. The other strain MTCC 532 was not found effective for hairy root induction in both *in vivo* and *in vitro* leaf. Like the case of internode, during co-cultivation period the leaf explant inoculated on ½ MS medium fortified with acetosyringone only resulted in production of hairy roots but ½ MS without supplemented with acetosyringone could not be able to develop a single hairy root from either type of leaves. Different infection time (15, 30, 45, 60 and 75 minutes) at different O.D. (0.086, 0.290 and 0.375) with co-cultivation period of 44-48 hours were examined for hairy root induction (Table 3). The initiation of hairy root was observed from midrib and side cut of infected *in vitro* leaves after 15 days of infection (Figure 1 h). Overall, at O.D. value 0.290 with 45 minutes infection time and 44-48 hours co-cultivation period using MTCC 2364 strain exhibited best results (approx. 89%, 3.1 hairy roots with a length of 2.94 cm) for hairy root induction (Table 2). The *in vivo* leaves failed to show any hairy root induction, rather they eventually became brown in colour and became necrotic (Table 3).

Table 3. Factors influence hairy root induction in *in vitro* leaves infected by MTCC 2364.

O.D.	Infection Time (in minutes)	Co-cultivation Period (in hours)	Average Hairy root induced (%)	Number of induced Hairy root (average)	Length of Hairy root (average)
0.086	15	44-48	0	0	0
	30		61.10^{c}	2.1^{bc}	1.97^{bc}
	45		77.75^{abc}	2.61^{ab}	2.66^{abc}
	60		49.99^{d}	1.83^{d}	1.77^{d}
	75		0	0	0
0.290	15	44-48	0	0	0
	30		0	0	0
	45		83.32^{ab}	2.55^{abc}	2.78^{ab}
	60		88.86^{a}	3.1^{a}	2.94^{a}
	75		22.21^{g}	1.83^{d}	1.55^{de}
0.375	15	44-48	16.66^{gh}	0.83^{g}	0.62^{f}
	30		38.88^{ef}	1.36^{ef}	1.97^{bc}
	45		0	0	0
	60		0	0	0
	75		0	0	0

Data pooled from 03 explants per flask, 02 flasks per replication and the experiment repeated 03 times (3*2*3=18). Each value is the mean from 3 replications. Mean values showed by different letters are significantly different at $p < 0.05$ (DMRT).

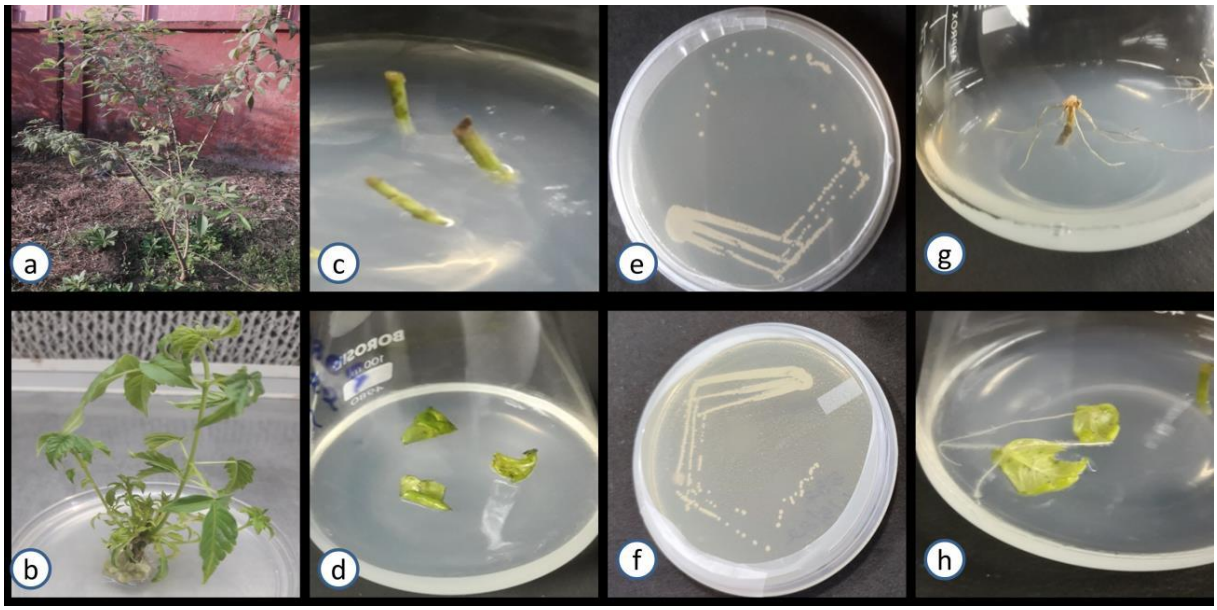

Figure 1. a: *In-vivo Vitex negundo* plant in the garden, **b:** *In-vitro* shoot culture in MS with BAP (2 mg/L) media, **c:** *In-vitro* internode infected by MTCC 2364 before hairy root induction, **d:** *In-vitro* leaves infected by MTCC 2364 before hairy root induction, **e:** MTCC 2364 grow in petriplate, **f:** MTCC 532 grow in petriplate, **g:** Hairy root induction from *in-vitro* internodes infected by MTCC 2364, **h:** Hairy root induction from *in-vitro* leaves infected by MTCC 2364.

4. DISCUSSION and CONCLUSION

Out of the two strains namely, MTCC 2364 and MTCC 532 employed in this hairy root induction experiment, the MTCC 2364 strain of *A. rhizogenes* was found effective for hairy roots induction, while the MTCC 532 strain was found ineffective in our experiment. Similar to this study, other researchers Muthiah *et al*. (2016) (*Bacopa monnieri*), Bathoju *et al*. (2017) (*Chlorophytum borivilianum*), and Bhagat *et al*. (2019) (*Rauwolfia serpentina*) also used these two types of strains for hairy root induction. However, in contrast to our study, in all these experiments, although MTCC 2364 was found to be effective for hairy root induction but the

strain MTCC 532 was more effective in term of percentage of hairy root induction, biomass, number and length of hairy root. On the other hand, MTCC 532 also works effectively for hairy root cultures of *Plumbago rosea*, *Rubia tinctorum*, *Arachis hypogaea*, and *Withania somnifera* (Brijwal & Tamta, 2015).

Different types of explants, *in vivo* leaf, internode, *in vitro* leaf and internode were used in this experiment, out of which, only *in vitro* leaf and *in vitro* internode explants responded to the induction of hairy roots. Of the two *in vitro* explants, the *in vitro* leaf performed better than the *in vitro* internode. Jesudass *et al*. (2020) (*Cucumis anguria*) also reported about the susceptibility of *in vitro* explant towards the infection of *Agrobacterium* strain which agrees with this result. Contrary to our result, Bhagat *et al*. (2019) got success in hairy root transformation in *R. serpentina in vivo* leaf explants. Swain *et al*. (2010) also proposed that stem internodes are significantly more sensitive than leaf explants, irrespective of the *Agrobacterium* strains used. But many of the researchers like Sajjalaguddam *et al*. (2016) (*Abrus precatorius*) and Bathoju *et al*. (2017) (*Chlorophytum borivilianum*) used *in vitro* explants like germinated seedling and shoot base respectively for hairy root induction experiment.

The O.D. value plays a crucial role for transformation and hairy root induction. Multiple investigations have reported that the *A. rhizogenes* and their densities have an impact on the rate of hairy root induction during transformation (Kumar *et al*., 2006; Shahabzadeh *et al*., 2014). All strains considerably enhanced the transformation frequency after identifying the best inducing O.D. (Majumdar *et al*., 2011). In our investigation, the best O.D. value at $O.D_{600}$ of the MTCC 2364 is 0.29 and 0.042 for the hairy root induction in *in vitro* leaf and internode respectively. Like our experiment different workers reported about different O.D. values of *A. rhizogenes* for optimum result for transformation. In *R. serpentina*, both MTCC 532 and MTCC 2364 showed 31% and 24% hairy root induction respectively at 0.6 optical densities of *A. rhizogenes* culture (Bhagat *et al*., 2019). According to the findings of Mahendran *et al*. (2022), different strains had different optimal induction concentrations. Further, lower bacterial concentrations resulted in lower transformation frequencies, whereas bacterial densities above the O.D. value 1.5 resulted in lower transformation rates due to severe necrosis. A higher cell density increased the T-DNA adherence to host cells, which resulted in browning of explants and a decrease in the frequency of hairy root induction.

In this experiment, we have tried some variable time (i.e., 40-44, 44-48, 48-52, 52-56 hours) of co-cultivation period whereas, the effective co-cultivation duration was standardised as 44-48 hours for hairy root induction. In corroboration to our result, Giri *et al*. (2001) as well as Brijwal and Tamta, (2015) also suggested about the 48 hours of co-cultivation duration as beneficial for hairy root induction for *Artemisia annua* and *Berberis aristate* respectively. Further, 48 hours of co-cultivation time was suggested by Srinivasan *et al*. (2023) for hairy root development in *Aerva javanica* by five different bacterial strains (ATCC 15834, R1000, LBA 9204, MTCC 2364, and MTCC 532).

Different infection times like 15, 30, 45, 60, and 75 minutes were studied and found that 60 minutes of infection time gave the best results for our explants; however, increasing the infection time up to 75 minutes in both *in vitro* leaf and *in vitro* internode decreased the rate of hairy root induction. The finding of Orlikowska *et al*. (1995) in the explants of safflower cv. and Kumar *et al*. (2023) in Plumbago zeylanica 'Centennial' reported a similar trend like our experiment. In *Carthamus tinctorius*, 30 minutes infection time was found suitable for higher transformation efficiency however, increasing the infection time up to 45 min caused a drop in effectiveness, which may be related to the explants' hypersensitive reaction to bacteria.

Gelvin (2000) explained that acetosyringone enhances the frequency of transformation of infected explants by activating *vir* genes. According to Brijwal & Tamta (2015), acetosyringone

in co-cultivation media not only enhanced explant transformation but also reduced the time needed for hairy root induction in contrast with acetosyringone free medium. They reported that in *Berberis aristata*, acetosyringone supplemented medium showed 72.22% transformation frequency whereas, acetosyringone free medium showed 61.11%. In this present hairy root experiment, ½ MS supplemented with acetosyringone (AS; 19.6 mg/mL) media effectively induces hairy roots whereas no hairy roots were observed from any type of the explants inoculated on ½ MS without addition of acetosyringone. Bhagat *et al*. (2019) and Moola *et al*. (2022) also suggested about the addition of acetosyringone in 125 µM for *Rauwolfia serpentina* and 100 µM for *Celastrus paniculatus* respectively for hairy root induction. However, Godwin *et al*. (1991) found that differences in transformation efficiency at various acetosyringone concentrations are significantly influenced by plant genotype and bacterial strains.

A protocol for hairy root transformation of *V. negundo* using *A. rhizogenes* strains MTCC 2364 was established. The *in vitro* leaf was found as most suitable explant for hairy root induction. However, this is a preliminary work and further research is required for molecular conformation analysis of transformed root, hairy root biomass enhancement, parameters optimization for production and enhancement of bioactive secondary metabolites of particular interest as well as designing of bioreactor for up-scaling of hairy roots of *V. negundo*. As *V. negundo* is a significant medicinal plant, hairy root cultures might be considered as a useful system for large-scale production of important secondary metabolites in general and those are located in the root in particular.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Bhaswatimayee Mahakur: Experimental designing, data recording and writing-original draft. **Arpita Moharana**: Helps in writing and data analysis. **Sanjay K. Madkami**: Helps in DMRT data analysis. **Soumendra K. Naik**: Reviewing and supervision. **Durga P. Barik**: Supervision and Validation of data.

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