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Research Article

Germline transformation of *Artemisia annua*L. plant via *in planta* transformation technology "Floral dip"

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ABSTRACT

The therapeutic efficacy of *Artemisia annua* L. is governed by artemisinin (ART), prevalently produced by *A. annua* extraction. Due to the modest amount of ART (0.01-1 %dw) in this plant, commercialization of ACTs is difficult. In this study, the floral-dip based transformation protocol for *A. annua* was developed to enhance expression of artemisinin biosynthesis genes and ART content. For dipping, the effective infiltration media components were optimized, and to obtain high transformation (26.9%) partially open bud stage capitulum of floral development was used. Hygromycin phospho-transferase (*hptII*) selection marker was used to validate the transformed T_1 progenies. The copy numbers of the transgene (*hptII*) in T_1 progenies were determined using a sensitive, high-throughput SYBR Green based quantitative RT-PCR. The results of the *hptII* transgene were compared with those of the low copy number, internal standard (*hmgr*). Using optimised PCR conditions, one, two and three transgene copies in T_1 transformants were achieved.

1. Introduction

Artemisia annua L. is an Asteraceae-family annual herb native to Asia. It has long been used to make fragrant wreaths, flavoured drinks, and essential oil distillation for perfumery and industrial usage. This plant has also been shown to have anti-hyperlipidaemic, anti-plasmodial, anti-convulsant, anti-inflammatory, anti-microbial, anti-cholesterolemic, and antiviral properties [1–3]. Additional clinical features of *A. annua* include hepatoprotective, antifungal, anticancer, antioxidant, anti-inflammatory, and anti-asthmatic qualities [4–11]. *A. annua* is currently considered one of the top 10 industrial crops in the contemporary world as a result of these findings [12].

Artemisinin(ART), a sesquiterpene-lactone with an endoperoxide bridge [13] is isolated from *A. annua* L. In 1970s, the function of artemisinin in the treatment of malaria was established with the rediscovery of this life-saving medication [14]. It has promising anti-malarial property [13,15,16] and part of artemisinin-based combination therapies (ACTs) to treat malaria. Together with antimalarial activity ART and its derivatives are used to treat various types of cancers, autoimmune disorders, diabetes and some viruses [17,18] including SARS-CoV-2 [19,20]. Being a therapeutic agent with a wide range of applications, ART has long been regarded as a promising multifunctional natural substance [21,22].

Profitable commercialization of ACTs is however, challenging due to the low content of ART (0.01-1% dry wt.) in *A. annua*. [23]. So, there is an urgent need to understand ART production dynamics and to increase its production in plants. The chemical synthesis of ART is difficult to perform and expensive because of the endoperoxide bridge [24]. Although, semi synthetic production of ART in yeast has also been developed [25,26], but due to some limitations the ART produced through this technology is not accessible to the poorest. To date, the main method of ART production is therefore, its extraction from *A. annua* L. Consequently, plant metabolic engineering has gained a lot of importance and being pursued to achieve a greater content of artemisinin in transgenic plants to lower the price of artemisinin [27–31].

Nowadays, the most extensively used method for *A. annua* transformation is the *Agrobacterium*-mediated transformation. It has proven to be a viable option for biosynthetic biology. *In vitro* raised plant transformation approach classically involves sophisticated regeneration methods that comprise multiple steps from *in vitro* culture to greenhouse conditions. At this point, in order to avoid the need for tissue culture phases in the regeneration of transgenic plants, we are introducing an alternative method i.e., the *Agrobacterium*-mediated transformation by "*in planta*" approach. This method yields genetically homogeneous

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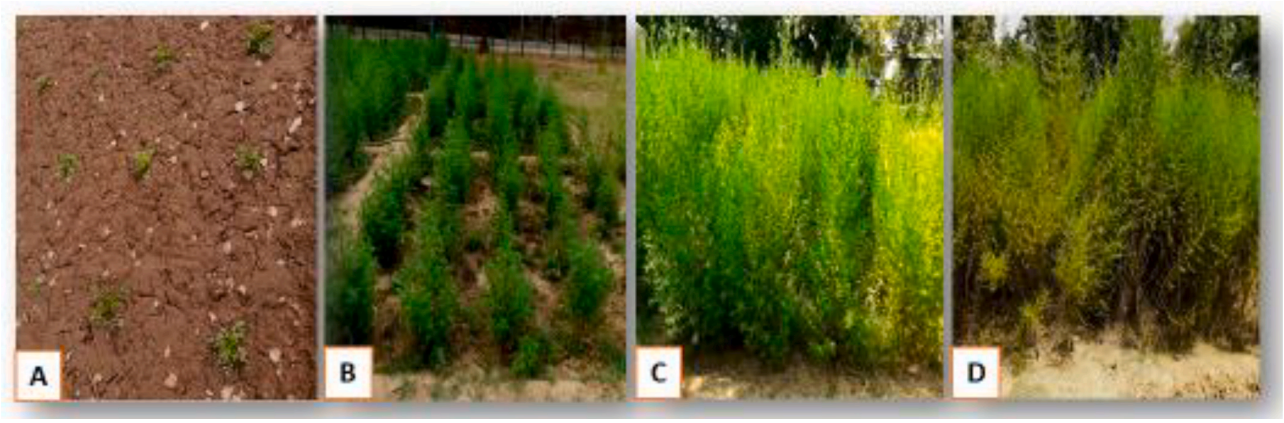


Fig. 1. Cultivation of *A. annua* plants for floral dip experiment. **(A)** One month old**; (B)** Three months old**; (C)** Five months old**; (D)** Eight months old *A. annua* L. plant under natural light condition in net house.

offspring with little soma-clonal diversity [32]. It is hypothesised that an *in planta* floral dipping event happens before or early in the floral development of the T_0 plant, resulting in male and female gametophytes that are similar. Thereafter, resulted in a large number of T_1 plants homozygous for the T-DNA insertion when self-fertilized. The infrequency of such lines, or their complete absence, suggests that the required transformation events are happening during germ-line development, after the divergence of male and female gametophyte cell lineages or potentially shortly after T_1 embryo fertilization [33].

Bechtold et al., 1998 [34] were the first to demonstrate the floral-dip transition in *Arabidopsis thaliana*, which was later refined by Clough and Bent, 1998 [32]. Later, using a similar approach, Curtis and Nam (2001) [35] reported the successful generation of transgenic radish and thereafter many more scientists that have reported the transgenic plant production of tomato [36], wheat [37], rapeseed [38], white sweet clover [39], camelina [40], flax [41], and rice [42]. Here, we for the first time, demonstrated the efficacy of the floral dipping method for the Asteraceae family member i.e., *Artemisia annua* L. with enhanced expression of artemisinin biosynthesis genes, which may possibly increase the artemisinin biosynthesis and its yield.

2. Materials and method

2.1. Plant material and growth conditions

The seeds of non-transgenic *Artemisia annua* L. plant (0.6-1% artemisinin) gifted by M/S Ipca Pvt. Ltd., Ratlam (M.P.), were germinated on soil in transgenic containment facility of Department of Biotechnology, Jamia Hamdard. After one month of seed germination the seedlings were transferred in natural light condition and grow them until its initiation of flowering stage (Fig. 1). The numerous numbered partially open, immature bud stage capitula were used for floral dipping.

2.2. Marker selection and cloning of gene of interest

The expression cassette containing multiple genes of ART biosynthesis (to be published in next report) including with hygromycin phospho-transferase gene as a selection marker were cloned in the nuclear transformation vector utilised in this work, which was created in silico. Each gene was placed under the control of its own promoter for effective expression. The CaMV 35S promoter was used to regulate the *hptII* gene, which confers resistance to selection marker gene i.e., *hptII*.

2.3. Agrobacterium tumefaciens strain, inoculum and infiltration medium

The *A. tumefaciens* strain *EHA105* harbouring plant expression vector Pcambia1300 with that of expression cassette was used for the transformation of *A. annua*. Streaking of EHA105 to YEM-agar plate along with 50mg/l kanamycin and 10mg/l rifampicin antibiotics was performed and incubate at 28°C in the dark for 2 days. After 2 days of inoculation monoclones were picked from the solid YEM plate and transfer it to the 2 ml of liquid YEM media with antibiotics of 50mg/l kanamycin and 10 mg/l rifampicin and incubate at 28°C and 120rpm for next 2 days i.e., the primary culture. Further it dilutes the 1 ml bacterial culture to 50 ml YEM medium with antibiotics of 50mg/l kanamycin and 10 mg/l rifampicin and grow the culture at 28°C (OD_{600}, 0.4-0.6). *A. tumefaciens* cells were harvested from freshly prepared culture by centrifugation at 4°C and 4000rpm for 10 min.. The cell pellet was resuspended in infiltration medium (pH-5.8) modified from Clough and Bent, 1998 [32] containing Murashig and Skoog (MS) medium supplemented with 5 % sucrose and 0.075% Tween-20 as well as in addition with 2mg/l Benzylaminopurin (BAP) and 50mM Acetosyringone in separate experiment.

2.4. In planta Artemisia annua L. transformation

On the day of floral dipping, the *Agrobacterium* cells were resuspended in 200 ml of freshly prepared infiltration medium and this bacterial suspension was incubated at room temperature in dark for 3 hours (for activating the cells) [29] with continuously stir in incubator shaker. The partially opened bud stage capitulum of *Artemisia* were selected and quietly wash it with sterilized double distilled water that would eliminate the unwanted surfactants. Followed by drying with tissue paper. For floral dipping, the pre-activated infiltration medium was added to a wide mouth flask and selected capitulum were inverted into this suspension such that all above-ground tissues were submerged, and capitulum were then removed after 3–5 mint of gentle agitation. The process of dipping was repeated 2 - 3 times, with approximately 12-15 days intervals between dipping with same capitulum. To retain humidity, the infiltrated capitulum were covered with thin paper bags. The paper bags were fastened with a needle to pass the light after two weeks and covered with the same paper bags till seed harvesting. After another 4-6 weeks of growth, when the inflorescences of the plants were brown and dry, the seeds were harvested by gently pulling the grouped inflorescences through the fingers over a sheet of clean paper.

2.5. Molecular analysis of transgenic Artemisia annua by PCR amplification

The harvested T_0 seeds were collected out in small Ziplock bags to let them dry completely and further germinated on soil in transgenic containment facility of Department of Biotechnology, Jamia Hamdard. After four weeks of seed germination, when few juvenile leaves were produced from T_1 progeny plants, genomic DNA samples were isolated

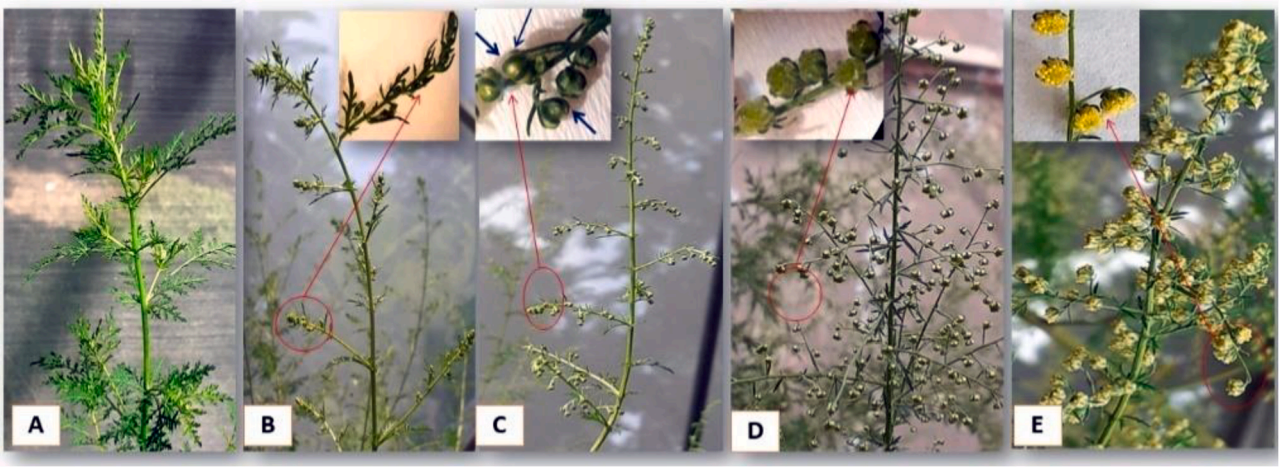


Fig. 2. Illustration of floral development stages in *A. annua* L. showing from A to E. **(A)** Green vegetative shoot; **(B)** Reproductive shoots with inflorescence buds; **(C)** The green capitulum stage with bracts of capitula; **(D)** The capitulum stage, that are partially open; **(E)** More advanced capitula with numerous florets.

and further used to perform direct PCR analysis to detect the presence of selection marker (*hptII)* gene by using gene specific primers, Forward *hptII* primer sequences: 5' -TCGGCCCAAAGCATCAG- 3', Reverse *hptII* primer sequences: 5'-CCGCAAGGAATCGGTCAATA- 3' to amplify the 150bp fragment of hygromycin phospho-transferase gene. Each PCR reaction was carried out in a 25µl (final volume) reaction mixture containing 10 x reaction buffer, 2.5 mM dNTPs, 10 nano moles of each DNA primer, 0.5µg template DNA and 3U/µl of Taq DNA polymerase. The reaction mixture was heated to 94°C for 3 min. to melt the template DNA, followed by 35 cycles of 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min. After 35 cycles, an additional final extension at 72°C for 10 min was performed to extend any premature DNA synthesis.

2.6. Determination of hptII gene copy number by quantitative real time-PCR

2.6.1. Genomic DNA extraction

Leaf samples from 27 transgenic and one non-transgenic plants were used to determine the copy number of plants bearing the *hptII* transgene. Using the CTAB technique, genomic DNA was isolated from 100 mg of fresh leaf samples. DNA purity was assessed based on the UV absorption ratio at 260/280 nm, while genomic DNA was quantified by UV absorption at 260 nm.

2.6.2. Quantitative RT- PCR reactions and conditions

All the primers used for copy number analysis were synthesized by IDT Primer Quest tool (http://eu.idtdna.com/primerquest/home/index) and analysed by Oligo Analyser (https://eu.idtdna.com/calc/analyzer). In this study we have chosen the *hmgr* gene as a low-copy number internal control (reference gene) for the *Artemisia* samples utilizing the comparative Ct method. Forward *hmgr* primer sequences: 5'-GAGGCAGTAATCACTGAAGA -3', Reverse *hmgr* primer sequences: 5'-TCCTGACCAGTGGCTATAAAGAC -3'. SYBR Green PCR Master Mix (Thermo Scientific, USA) was used to perform quantitative RT-PCR experiments in a Biorad CFX96 real time PCR on 96 well plates in a final volume of 10µl. Three technical replications of 10-µL reactions containing 10 nM gene-specific primers in 1× SYBR Green PCR Master Mix, plus dilutions of genomic DNA were run on a qRT-PCR. Experimental conditions were as follows; initial denaturation (94°C for 3 min), amplification and quantification program (94°C for 1 min, 55°C for 30 s, 72°C for 1min with a single fluorescence measurement) for 35 cycles, melting curve program (70-95°C with a heating rate of 1.0°C per 5 sec and a continuous fluorescence measurement) to verify primer specificity and finally a cooling step to 37°C for 10 min. Average PCR efficiency(E =10ˆ(-1/slope) – 1) for each amplicon and starting quantities(SQ) values were calculated by Omar et al., 2008 [43]. Gene copy number was calculated by using *hmgr* as a 2-copy number [10] reference gene and represented as 2*(SQ)-target gene/(SQ)-reference gene [44,45].

3. Results and Discussion

This study was started in order to offer such an assessment and to pinpoint changes that support high throughput transformation. The transformation of *Artemisia* through a tissue culture phase has been successful, but it has several limitations. Tissue culture allows for the selection of single transformed cells that are regenerated in a whole plant, reducing the production of genetic chimeras. Tissue culture, on the other hand, causes somaclonal variation due to epigenetic effects or chromosomal rearrangements [46,47]. In subsequent generations, biolistic (Particle bombardment) can also result in multiple T-DNA insertions and gene silencing [48]. Recently in 2021, *Agrobacterium* mediated transient expression system was developed for *Artemisia* to reduce gene silencing and reliance on tissue culture, but the system's limitation is that it is not intended to integrate the gene of interest into the genome, making it unable to produce stable line transformants [49]. Thus, a relatively convenient approach has been made easier and much more practical by improvements and simplifications to *Artemisia* transformation through floral dipping. To the best of our knowledge, this has proven to be the most effective method for transforming *Artemisia* plant. It has done by optimizing different stages of floral growth and standard infiltration medium components. In order to validate the transgene integrity in T_1 progenies, a quantitative RT-PCR assay was described for estimating the copy numbers of transgene *hptII* in transgenic *A. annua* by comparing them to the endogenous reference gene i.e., 3-hydroxy-3-methylglutaryl coenzyme A reductase(*hmgr*) [43,44,50,51].

3.1. Examination of floral development stages

It suggests that there is an optimal period of floral development for either *Agrobacterium* access to target tissue or that tissue's susceptibility to transformation when the greatest number of viable flower buds are present, largely in appropriate phases [32]. In present study different stages of floral development of *Artemisia* were analysed to optimise the favourable capitulum stage for floral dipping at subsequent time period of plant growth. The first floral dipping has been taken place at the partially open, numerous number bud stage capitulum i.e., Immature inflorescence bud stage (Fig. 2, (C), (D)) rather than in Capitula with florets that are closed (Fig. 2, (B)) or even in the mature inflorescence stage (complete open) (Fig. 2, (E)). In a similar manner, Zale et al. in

Table 1
Artemisia annua L. inflorescence transformed with different compositions of Agrobacterium Infiltration media.

Infiltration media	MS basal medium	Sucrose (5%)	BAP (1mg/ml)	Acetosyringone (1M)	Tween-20 (0.075%)
I	1/2MS	+	-	-	+
II	1/2MS	+	-	50mM	+
III	1/2MS	+	2mg/l	-	+
IV	MS	+	-	-	+
V	MS	+	-	50mM	+
VI	MS	+	2mg/l	-	+

+, present; -, absent
Always make fresh on the day of floral dipping

2009 took the early to mid-boot stage, pre-anthesis wheat spikes for floral dipping [37]. It is however, possible that the closed capitulum may block the entry of *Agrobacterium* and more mature flowers had closed locules that also prevented *Agrobacterium* from reaching the growing ovules and megaspores, it is hypothesised that no transformants were found in flowers that were more mature at the time of inoculation. Pistils that are only at partially open stage give *Agrobacterium* fast access to the female reproductive tissues, which are necessary for efficient transformation [52]. Furthermore, when Desfeux and colleagues performed the floral dip transformation experiments with the CRABS-CLAW (*crc*) mutant of *Arabidopsis* with open pistils throughout the flower development phases and the near-isogenic parental line Ler-0, they found that the *crc-1* mutant line transformed at a 6-fold higher rate than wild type Ler-0. However, employing the floral dip approach to transform soybeans fails because their pistils were closed during the early stages of floral development [33]. Thus, open pistils are favourable for floral dipping. It appears that the transformation of growing ovules and delivery of *Agrobacterium* to the interior of the gynoecium are essential criteria for successful transformation by these techniques.

3.2. Examination of effective infiltration medium for floral dipping

This is the first study to our knowledge in which the *in-planta* transformation approach was used to transform *Artemisia*. So, the need of the hour was to optimise the effective infiltration media for successful transformation. We have chosen the infiltration medium in six different compositions (Table 1) with *A. tumefaciens* strain *EHA105*, containing Pcambia1300 vector construct. Here, we looked into the relative importance of different media elements in the transformation of *Artemisia*. According to clough and bent, 1998 the standard infiltration medium used to transform *Arabidopsis* plant, contained 1/2 MS medium, pH 5.7, 44 nM BAP, 5% sucrose, 0.005% Silwet L-77. They examined the contributions of these media components and concluded that *Arabidopsis* transformants were made at a high rate (0.5-3 %) by dipping blooming plants in a solution containing sucrose (or glucose) and the surfactant (Silwet L-77). They said that sucrose was an essential component of the inoculation medium and when sucrose was removed, there were few or no transformants; apart from sucrose, surfactant was the only other essential component of the inoculation medium. They reported that the absence of the surfactant (Silwet L-77) reduced transformation efficiency three to four-fold, whereas a four-fold rise in Silwet L-77 (to 0.02 percent) nearly doubled transformation efficiency. Furthermore, they also found that the plant tissue culture media, pH modifications, plant growth regulators, and *Agrobacterium* growth or use at certain culture densities were not required for successful, high efficiency transformation [32]. In contrast, Ratanasut et al. (2017) reported that in the study of rice transformation, the inoculation medium without a pH adjustment or without MS slightly reduced the transformation efficiency, and the removal of sucrose and surfactant (Tween-20) from the inoculation medium also played a significant role in lowering the transformation efficiency [42], much like Clough and Bent. So, the present study was designed by analysing all the previous studies on floral dip transformation and here, we used the Murashig Skoog medium (MS) salts with vitamins in two different ways to optimise the effective infiltration media component, one with full MS and the other with half MS basal medium composition, to look out whether it is low or high MS salts concentration feasible for high efficiency and we discovered that in the case of *Artemisia*, the half strength MS medium provided slightly higher transformation efficiency than the full MS medium. (Table. 2; Fig. 4-A). It is yet unclear why this is the case, but it is possible to hypothesise that infiltration media with low salt concentrations assist to minimize osmotic stress and promote the growth of *Agrobacterium* cells in suspension, which would improve the efficiency of transformation. The supported study of Mukherjee et al. 2010 on effect of medium composition shown that when grape rootstock was cultivated on MS with half of the nitrate medium, shoot proliferation increased [53]. Aside from that, Fakhrul et al. 2014 indicated that manipulating macronutrients in MS medium might alter the development of *Stevia rebaudiana* [54]. As Clough and Bent (1998) discovered that for the floral-dip transformation in *Arabidopsis*, employing an infiltration medium without the surfactant (Silwet-L77) significantly reduced transformation efficiency, but higher concentration of the surfactant (Silwet-L77) drastically enhanced it [32]. In this work, tween-20 was used as a surfactant in the composition, as Li et. al., recently (2021), shown that all three surfactants (Silwet L-77, Tween 20, and Triton X-100) were able to increase the efficiency of transient transformation in *A. annua* [49]. In one experiment of clough and bent, removing BAP from the infiltration media reduced transformation rate by twofold, but had no effect in a subsequent trial. And in present study the BAP plays an important role in efficiency improvement like with half MS +Tween-20, addition of 2mg/l of BAP increased the transformation efficiency from 17.7% to 19.3% and in case of full MS + Tween-20 addition of same 2mg/l of BAP increased the transformation efficiency from 11.5% to 13.6%. Interestingly, we found that including with BAP, acetosyringone also play important role to improve the transformation efficiency from 17.7% to 26.9% (in half MS + Tween-20 combination) and from 11.5% to 21.9% (in full MS + Tween-20 combination) indicating that acetosyringone was more prominent than BAP for efficient transformation process (Fig. 4-A). These findings contrast a previous study in which acetosyringone did not significantly improve the transformation efficiency of *A. annua*, which might be related to the use of different explants [29]. Supported studies on various crops, reported that no GUS expression was seen in the absence of acetosyringone in wheat, and the transformation efficiency was 0% and when it was present in the media, GUS expression was 90% [55]. Similarly, when acetosyringone was added to the medium for the transformation of cotton, the average number of kanamycin-resistant calluses were 2-3 times greater [56]. Acetosyringone has been proven to be effective even in tobacco transformation, where it boosted efficiency thrice when incorporated in

Table 2
Effect of various sets of infiltration media composition on rate of transformation.

Infiltration	Media composition	T_1 progeny plants tested for direct PCR amplification	Hpt +ve progeny
I	1/2MS+Tween-20 (0.075%) +BAP(2mg/l)	31	06
II	1/2MS+Tween-20 (0.075%)	45	08
III	1/2MS+Tween-20 (0.075%) +Acetosyringone (50mM)	52	14
IV	MS+Tween-20(0.075%) +BAP(2mg/l)	22	03
V	MS+Tween-20(0.075%)	26	03
VI	MS+Tween-20(0.075%) + Acetosyringone (50mM)	41	09
	Total	217	43

Fig. 3. Steps involve in *Artemisia annua* floral dipping. **(A)** *A. annua* L. inflorescence; **(B)** Microscopic view(10x) of Immature inflorescence bud used for floral dipping; **(C)** Dipping of green, immature and closed bud stage inflorescence in to infiltration medium; **(D)** The dipped (infected) inflorescences were covered with paper bags; **(E)** Treated progenies(T_1); **(F)** Positive transformants were selected and grown to transgenic containment zone.

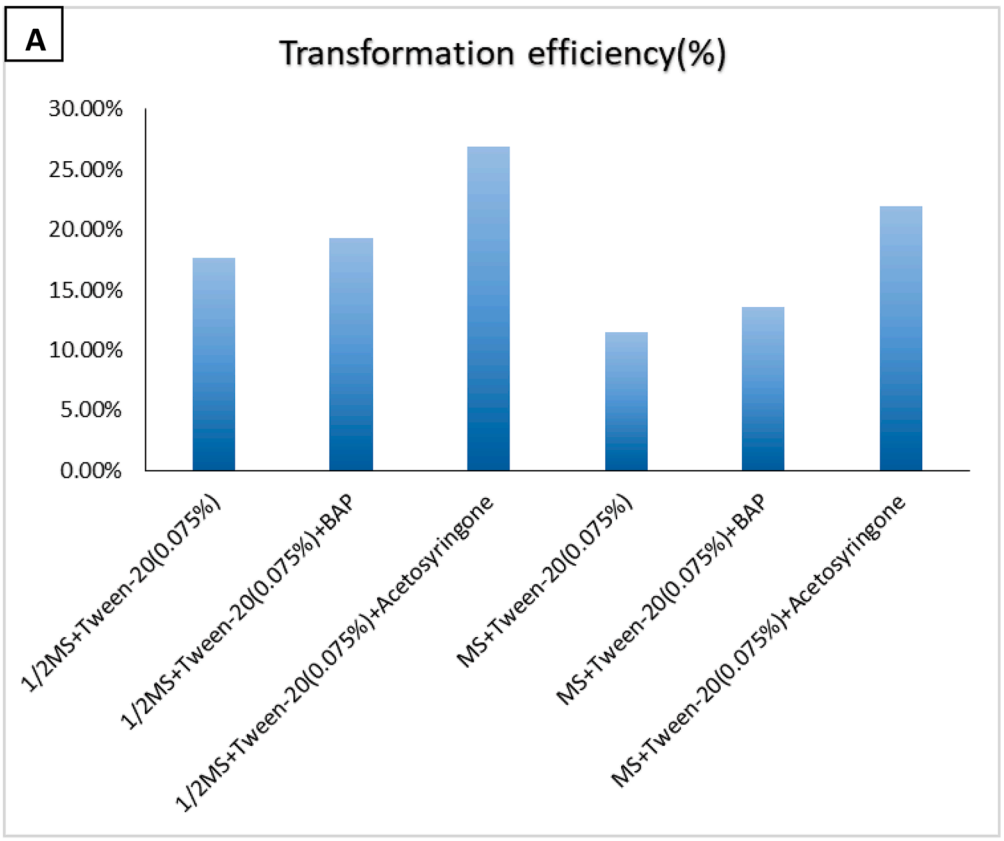
co-cultivation medium [57]. These studies thus, indicates that the transformation procedure required acetosyringone, which is largely required to improve overall transformation efficiency.

3.3. Transgenic A. annua plant production by high efficiency transformation

Floral-dip transformation under modified circumstances from Clough and Bent, 1998, was performed to test the feasibility of generating transgenic *Artemisia* through this method. For this, the pre-anthesis capitulum i.e., the partially open bud stage capitulum as shown in Fig. 2, (C), (D) have taken and immersed it in to the infiltration medium (Fig. 3 C). At least 20-30 *A. annua* plant capitulum were treated in order to develop large number of transgenic lines. According to supporting study (Desfeux et. al., 2000), the efficacy of transformation following gentle dipping of inflorescences into *Agrobacterium* solution revealed that the transformed tissues were exposed at or near the plant's surface. Potentially, the germinated pollen tubes transport *Agrobacterium* from the stigma to mature ovules persists [33]. It does not need to complete dark condition after dipping, but strong light might be reducing the infection rate of *A. tumifacience* [58]. As a result, covering of dipped inflorescence with thin paper bags to retain humidity during the co-cultivation phase was advantageous, with the idea that humidity may be equally significant in maintaining host tissues in an accessible configuration. Similar toxample, the transformation efficiency of Arabidopsis this, Zale et al. (2009) used plastic paper bags to ensure high humidity in dipped inflorescence of wheat [37]. However, when appropriate light was not passed in treated florescence after 3-4 days of co-cultivation, there was no seed development in that dipping inflorescence (data not shown). So, after 3-4 days of co-cultivation, paper bags were pinched with a needle to let natural light to get through for appropriate seed development. Another cause for high efficiency transformation in *Artemisia* might be the repeated (2-3times) administration of *Agrobacterium,* which boosted transformation rates and overall transformant output. It is consistent with prior findings that the number of transformants acquired on a plant may be enhanced by a second floral-dip application of *Agrobacterium* around 1 week after the initial dipping [32], suggesting that *Agrobacterium* survives for a short duration at levels high enough to achieve respectable rates of transformation in freshly emerging flower buds. For all six different infiltration media treatments, a high number of T_0 seeds were harvested, and T_1 seedlings were screened using a selection marker-based PCR amplification. A total of 217 T_1 progeny plants were screened by direct PCR analysis with selection marker (*hptII*) specific primers, in which 43 different transgenic lines were generated with overall transformation efficiency of 19.82% (Table. 2, Fig. 4-B) and survival rate was of 67.44%. However, the success of transgenic plant development from the floral-dip transformation differed by plant type. For example, the transformation efficiency of *Arabidopsis* can reach up to 3% [32], whereas the transformation efficiencies of *Raphanus sativus* L. longipinnatus [35], *Oryza sativa* [42] and *Solanum lycopersicum* [36] were 1.4%, 1.4% and 12% respectively.

3.4. Transgene hptII copy number estimation by comparison to the endogenous hmgr gene

To determine transgene copy number in transgenic *Artemisia*, a simple, efficient quantitative technique i.e. qRT-PCR was employed. Transgene copy number is measured relative to an endogenous reference gene in this methodology, and the design is such that any reaction variances attributed to initial template DNA amount are normalised. We



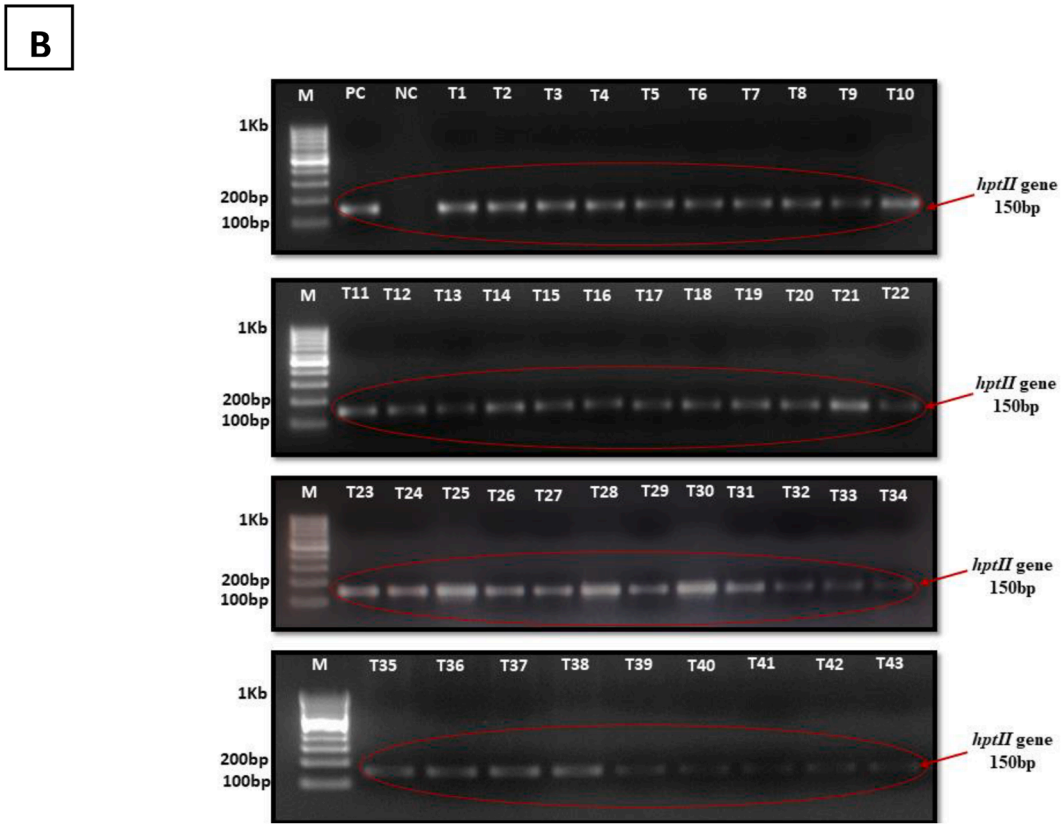


Fig. 4. A; Transformation efficiency of *A. annua* L. in different infiltration media composition. **B;** PCR amplification of *hptII* gene(150bp) in transgenic plants. The templates used for PCR amplification: lanes T1-T43, having the transgenic plant genomic DNA sample; lane NC, negative control having untransformed plant genomic DNA sample; lane PC, positive control having plasmid vector DNA sample. Lane M, 1 kb DNA ladder.

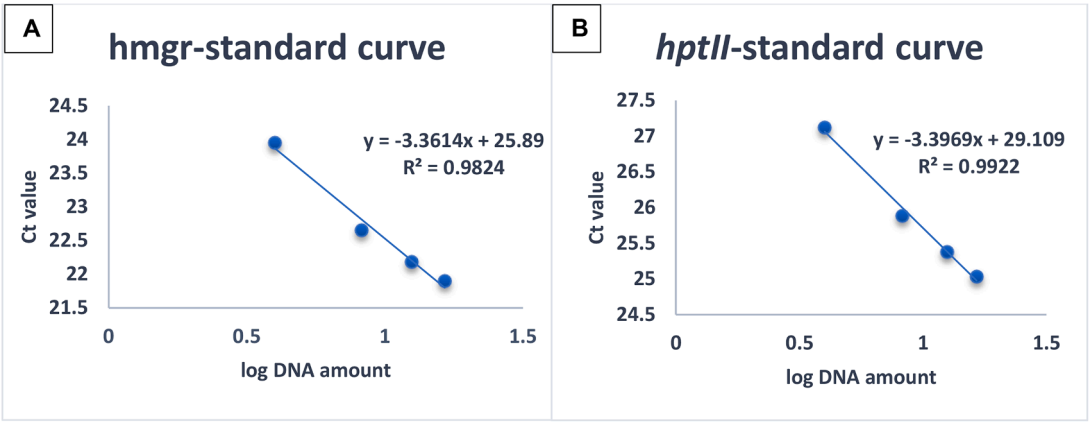


Fig. 5. A, B; Standard curves of endogenous *hmgr* and transgene *hptII* genes respectively. Calculated Ct values were plotted against the log ng total DNA(SQ). Each sample was run in three replicates.

used *hmgr* gene as an endogenous control since prior research had revealed that the *hmgr* gene is present in two copies [10], and we hypothesised that it would be acceptable for use as an endogenous reference gene in this work. Li et al., 2004 selected *puroindoline-b*, a 2 copy endogenous reference gene, to measure transgene copy number in transgenic wheat [59]. A pair of primers for the transgene *hptII* and endogenous *hmgr* present in transgenic *Artemisia* plants were designed for quantitative real time PCR (mentioned in Section 2.5 and 2.6.2 respectively). In this method, amplification of the transgene of interest *hptII,* was compared with that of an endogenous *hmgr* gene. The two genes were amplified in the same reaction simultaneously and identical conditions were achieved. DNA was prepared from the transgenic plants as well as the non-transgenic plants (control). In order to create a standard curve for the endogenous *hmgr* and the transgene *hptII*, genomic DNA from one of the transgenic lines was used, as Mason et al., 2002 described [60]. The standard curves were calibrated using four concentrations of genomic DNA (4, 8.25, 12.5, and 16.5 ng per reaction) from the selected transgenic line. Using these standard curves, the endogenous gene and the transgene were both relatively measured. As a negative control, a no-template control (NTC) was also established for the investigation. The correlation coefficients of the standard curves were 0.982 and 0.992 for the endogenous *hmgr* and the transgene *hptII*, respectively (Fig. 5)

3.5. Validation of the standard curves and PCR efficiency

The making of a standard curve is one of the crucial steps in figuring out how many copies of the transgene are present in any quantitative test. A standard curve should ideally be created using one of the transgenic lines whose copy number had already been confirmed using Southern blot analysis. Such a transgenic line was not available in our study. So, based on standardised curves from quantitative RT-PCR analysis of serial standard DNA dilutions of the *hmgr* and *hptII* genes, transgenic copy number estimates were established. Real time assays were used to test serial dilutions of genomic DNA from one of the transgenic plants. The PCR efficiency was measured by graphing the common logarithm of a DNA dilution series (4, 8.25, 12.5, 16.5 ng) against the Ct values of these dilutions. In the standard dilutions, these efficiencies were 98 % for endogenous *hmgr* and 97 % for transgene *hptII*, showing that endogenous and transgene amplifications happen at fairly similar rates and with high efficiencies. This is consistent with previous research in which PCR efficiencies of transgene and endogenous reference gene were identical or very close [51,61]. According to Ginzinger et al., 2002 the ideal slope should be -3.32 for achieving 100% PCR efficiency [62]. The PCR efficiencies obtained in this investigation were functional and precise enough to calculate the initial quantity of unknown transgenic samples. Similar to that of Yang et. al., (2005) and Omar et al., (2008), the standard curve was constructed three times to validate the quantification accuracy even further [43,51]. The repeatability of this qRT-PCR technique was determined with four *Artemisia* DNA dilutions, 4, 8.25, 12.5, and 16.5 ng, as shown in Table. For the endogenous *hmgr*, Ct values ranged from 21.9 to 23.95, CV percent values ranged from 0.65 to 1.4, and SD values ranged from 0.15 to 0.31 (Table. 3). The results for the transgene *hptII* were Ct (25.04 to 27.12), CV percent (0.95 to 2.39), and SD (0.25 to 0.62). The CV percent and SD values from these experiments were relatively small, showing that the qRT-PCR assay was stable and reproducible.

Table 3
Quantitative Real-Time PCR Reproducibility of the Replicate Standards amount (ng) of *A. annua* Genomic DNA for endogenous *hmgr* and transgene *hptII*.

Reference gene-*hmgr*						
DNA amount(ng)	**Ct-1**	**Ct-2**	**Ct-3**	**Mean**	**SD* (%)**	**CV** (%)**
4	23.79	24.1	23.96	23.95	0.15	0.65
8.25	22.85	22.45	22.67	22.65	0.20	0.88
12.5	22.43	21.94	22.16	22.18	0.24	1.11
16.5	22.2	21.6	21.79	21.9	0.31	1.40
Target gene-*hptII*						
DNA amount(ng)	**Ct-1**	**Ct-2**	**Ct-3**	**Mean**	**SD* (%)**	**CV** (%)**
4	27.07	26.89	27.4	27.12	0.26	0.95
8.25	25.73	26.56	25.35	25.88	0.62	2.39
12.5	25.07	25.84	25.25	25.38	0.40	1.59
16.5	24.95	25.37	24.8	25.04	0.29	1.18

*Standard deviation; **coefficient of variation

3.6. Estimation of hptII copy number in T_1 transformants

In this study, a relative quantitative approach was used to determine the *hptII* copy numbers, combining two absolute quantification reactions—one for the target specific gene and the other for the endogenous reference gene. As a result, we contrasted the quantitative outcomes of the *hmgr* endogenous gene with those of the *hptII* transgenic. In order to compare the experimentally obtained levels in each transgenic *Artemisia* sample with the standard curves for the transgene *hptII* and the endogenous *hmgr* gene, the amount of the transgene was divided by the amount of the endogenous gene. Czechowski et al. (2016) used qRT-PCR to determine the copy number of the *cyp71av1* gene in a hybrid variety of *A. annua,* using *squalene synthase (SS)* as a single-copy reference [44]. Similarly, Catania and colleagues used the same method in 2018 to

Table 4
Quantitative RT- PCR Estimates of Copy Number for *hptII* Transgene

T_1Transgenic lines	*SQ (REFERENCE)	*SQ (TARGET)	(2*SQ-T/ SQ-R)	Copy number
F191	1.77 ± 0.116	0.89 ± 0.005	1.01	**1**
F198	1.39 ± 0.055	1.03 ± 0.002	1.48	**1**
F202	1.89 ± 0.156	0.92 ± 000	0.97	**1**
F203	1.77 ± 0.038	0.66 ± 0.094	0.75	**1**
F218	1.45 ± 0.031	0.64 ± 0.107	0.88	**1**
F219	1.29 ± 0.159	0.59 ± 0.103	0.91	**1**
F220	1.49 ± 0.072	0.71 ± 0.128	0.95	**1**
F221	1.52 ± 0.075	0.67 ± 0.109	0.88	**1**
F222	1.10 ± 0.217	0.71 ± 0.033	1.29	**1**
F223	1.50 ± 0.091	0.50 ± 0.146	0.66	**1**
F224	1.28 ± 0.145	1.12 ± 0.057	1.75	**2**
F225	1.44 ± 0.150	1.17 ± 0.119	1.63	**2**
F226	1.43 ± 0.038	0.82 ± 0.095	1.15	**1**
F227	1.40 ± 0.028	1.25 ± 0.010	1.79	**2**
F228	1.38 ± 000	1.23 ± 0.004	1.78	**2**
F229	1.21 ± 0.066	2.04 ± 0.013	3.37	**3**
F230	1.72 ± 0.141	0.91 ± 0.036	1.06	**1**
F231	1.34 ± 0.091	1.19 ± 0.020	1.78	**2**
F232	1.33 ± 0.038	1.34 ± 0.033	2.02	**2**
F233	0.97 ± 0.032	1.14 ± 0.019	2.35	**2**
F234	1.13 ± 0.013	0.98 ± 0.051	1.73	**2**
F235	1.49 ± 0.072	1.30 ± 0.042	1.74	**2**
F236	1.93 ± 0.131	0.52 ± 0.001	0.54	**1**
F237	1.52 ± 0.126	0.69 ± 0.051	0.91	**1**
F239	1.22 ± 0.077	1.18 ± 0.067	1.93	**2**
F240	1.54 ± 0.211	0.82 ± 0.063	1.06	**1**
F241	1.7 ± 0.144	0.82 ± 0.028	0.96	**1**
Control (Non-Transgenic)	2.01 ± 0.091	0.02 ± 0.022	0.01	**0**

estimate the *amorpha-4,11-diene synthase (AMS)* gene copy number in transgenic *Artemisia* [45]. In the present study, a total of 27 unique transgenic lines of *Artemisia* were evaluated for copy number analysis, and the results revealed that ten samples have two copies, one samples have three copies, sixteen samples have one copy of the transgene (Shown in Table. 4).

*SQ=Starting Quantity (mean ± standard error)

These findings independently verified the previous findings of Zale et al., 2009, who concluded that the floral dip can be used to extract low-copy number transformants in wheat without the use of tissue culture [37]. It is as accurate as Southern blotting for low copy transgenic insertions and allows for significantly more accuracy in identifying medium and high copy transgene insertions than blotting methods. In the present study, SYBR Green was used instead of fluorescent probes for detection, making this approach more flexible to novel genes of interest and less expensive. Similarly, Li et al., 2004 concluded that real-time PCR not only reduces the amount of plant tissue required, but also allows for the determination of transgene copy number at an early stage of plant growth [59]. As a result, real-time PCR may provide a practical method for early detection of low or single copy transformation events.

4. Conclusion

The composition of 1/2MS, Tween-20(0.075%) and Acetosyringone (50mM) generated the highest number of transgenic lines with transformation efficiency of 26.9% that is unexpectedly high as earlier reported studies. *In planta* transformation allows the integration of genes of interest in *A. annua* genome in comparatively trouble-free manner. It requires little labour, inexpensive apparatus, and only a few specialised chemicals, and even non-specialists can accomplish it successfully. It is easily producing large number of independent *A. annua* transgenic lines with higher transformation efficiency than the current available methods. This could be a valuable platform for making transgenic *A. annua* plants using the floral-dip transformation approach. The results of qRT-PCR for transgene copy number quantification were highly consistence with those of Southern blot analysis. Furthermore, the high sensitivity and efficiency of qRT-PCR allowed us to analyse as much samples and quantify the transgene copy number more rapidly and correctly.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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