Journal of Scientific & Industrial Research
Vol. 81, October 2022, pp. 1019-1028
DOI: 10.56042/jsir.v81i10.59665

In vitro Direct Regeneration and *Agrobacterium Tumefaciens* mediated *in planta* Transformation of *Ocimum sanctum L.*

Sana Khan[1,2], Zakir Husain[2] & Laiq ur Rahman[1,2]*

[1]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201 002, Uttar Pradesh, India

[2]CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow 226 015, Uttar Pradesh, India

Received 28 January 2022; revised 23 June 2022; accepted 08 September 2022

An *in vitro* regeneration system for propagation has been successfully developed for a valuable medicinal and aromatic plant '*Ocimum sanctum* L'. In the present study, petiole explants, from *in-vitro* grown cultures of *O. sanctum,* was used for direct regeneration. The developed protocol employed 98% of regeneration frequency in addition to 9.6 shoots per explant when cultured on Murashige and Skoog (MS) medium fortified with 3 mg/L benzylamino purine (BAP) and 1 mg/L Naphthalene acetic acid (NAA). Furthermore, *Agrobacterium tumefaciens* Mediated genetic Transformation (ATMT) protocol (transient and stable) was also developed using LBA4404 strain harboring pBI121 with *uid-A* and neomycin phosphotransferase genes. The regenerated transformants were shifted on MS with kanamycin (50 mg/L) and afterwards placed on the half-strength MS medium. The validation was done through polymerase chain reaction (PCR) with neomycin phosphotransferase-II (*npt-II)* & β-glucoronidase (*uid-A)* gene primers. The maximum stable transformation frequency of 70% ± 0.35 was achieved. Hence, it is apparent that the established protocols *i.e. in vitro* direct regeneration and ATMT are appropriate for integrating novel enzymes/genes through high throughput techniques such as gene tagging, and targeted gene replacement to modulate the primary as well as secondary metabolic flux towards desired agronomic product or trait *in planta.*

Keywords: *Gus-A*, Kanamycin, MAPs, *Npt-II*, *O. tenuiflorum*, Petiole

Introduction

Ocimum sanctum L. (*O. tenuiflorum*) is a valuable sacred medicinal, and aromatic plant commonly called as "holy basil" or Tulsi.[1] It is widely used in ayurvedic, traditional, and Unani system of medicine.[2,3] The sacred tulsi herb is therefore named differently by people; some says as 'The Incomparable One', some says, 'The Queen of Herbs' for more than a decade and also used to worship for over 3000 years.[4] The bioactive phyto-constituents present in *O. sanctum* are a rich repository of tannins, alkaloids, flavanoids, oligosaccharides, phytates etc. Several phytochemicals such as carvacrol limatrol, eugenol, euginal, urosolic acid,[5] caryophyllene, and anthocyans[6] are some of the principal ingredients with therapeutic and traditional values since ages. The essential oil derived from *O. sanctum* showed efficacies as cardioprotective, analgesic, anti-fertility, anti-diabetic, anti-microbial, anti-cancer, anti-fungal, anti-spasmodic, adaptogenic properties, and so on.[6]

Despite having enormous pharmacological utilities, the underpinning pathways in *O. sanctum* remains insufficiently understood due to the lack of genetic transformation protocol. As a prelude to increasing the production of desired secondary metabolites through targeted breeding programs, the transcriptomic and genomic data needs to be explored and thus an efficient regeneration and transformation protocol is urgently required. Yet, few of the transformation protocols have been reported such as (i) in *O. gratissimum* where only 20% ± 0.7 stable transformation frequency was achieved[7]; while (ii) in *Ocimum basilicum* and iii) in *O. citriodorum*; only transient expression system was established by Deschamps and Simon.[8]

Therefore, for the first time, an efficient and rapid direct *in vitro* regeneration and ATMT protocol (transient as well as stable) has been reported in '*O. sanctum' (cim-ayu).* The established protocol is highly efficient and reproducible in the rapid regeneration of transgenic plantlets with a stable expression system. The method could be used as a potential tool in developing improved varieties with high therapeutic and economic value. Isolation and characterization of novel genes would also be

*Author for Correspondence
E-mail: l.rahman@cimap.res.in

done in order to modulate the desired transcriptional/translational regulons, which paves the way for further advancement in engineering of agronomic traits in related species.

Materials and Methods

Direct Regeneration and Multiple shoot Induction in *O. sanctum*

Seeds of *O. sanctum* were collected from the National gene bank of medicinal and aromatic plants (NGBMAP) at CSIR-CIMAP, Lucknow. The seeds were surface sterilized using 70% ethanol and 0.1% of $HgCl_2$ for 60 s. Afterwards, the seeds were washed with double distilled autoclaved water and shifted on MS medium[9] supplemented with sucrose and agar [3% (w/v) & 0.8% (w/v) respectively] having pH of 5.8 ± 0.5 for further growth and development.

After 4–5 week of establishing *in vitro* culture of *O. sanctum*, several explants including hypocotyl, petiole and cotyledonary leaf were excised and used for direct *in vitro* regeneration studies. The explants were placed on modified MS medium in combination with BAP (0.5 to 3 mg/L), and NAA 0.5 to 1.5 mg/L. The data was recorded after 4–5 weeks of culture inoculated on shoot induction medium (SIM). The regenerated flasks/petriplates were placed at culture racks fitted with fluorescent lights (3000 lux) and maintained at 25 ± 5°C. Afterwards, *in vitro* proliferated explants (1–1.5 cm) were shifted to basal MS medium and ½ MS for elongation & root induction, respectively. Further, the rooted plantlets having 5–6 leaves were thoroughly washed to remove agar and acclimatized and then hardened into potting soil containing equal ratio of vermiculite and sand. The plantlets were shifted to glass house for further growth and development. Out of several explants, petioles were considered best for further transformation experimentations as the highest proliferation was achieved from petioles.

Optimizing Parameters for *A. Tumefaciens* Mediated Genetic Transformation Protocol in *Ocimum sanctum L.*

Kanamycin Concentration

The optimal concentration of kanamycin was optimized in order to screen out the pseudo transformants and thus a sensitivity test was carried out in *O. sanctum*. The petioles were kept on SIM supplemented with different kanamycin concentrations (10–60 mg/L). The petioles that were cultured on SIM without kanamycin served as a control experiment. The data was collected after 4–5 weeks of culture.

Agrobacterium Tumefaciens Strain, Transformation Vector and ATMT

A. tumefaciens strain LBA4404 harboring binary vector pBI121 with *uid-A* gene (β-glucuronidase) and neomycin phosphotransferase II (*npt-II)* were used for genetic transformation study in *O. sanctum*. Both genes are present on T-DNA and driven by CAMV-35S promoter and Pnos promoter (nopaline synthase promoter) respectively.

A. tumefaciens was grown in Yeast extract peptone (YEP) medium containing streptomycin (250 mg/L), rifampicin *(*25 mg/L), kanamycin (50 mg/L), and kept on a rotary incubator/shaker (200 rpm) at 28 ℃ for overnight. *A. tumefaciens* cells were harvested [$OD_{(600)}$ 0.6] by centrifugation at 5,000 rpm for 10 min, and pellet was resuspended in equal volume of MS liquid medium. The explants were immersed in an agro bacterial infection medium containing AS (0–400 μM), Tween-20 (0.001–1%) and kept on a shaker (100 rpm) for 0–60 min. The infected explants were then blotted dry onto a sterile filter paper and placed on co-cultivated medium (CCM) for 48 hrs in the dark. Afterward, the treated explants were placed onto the SIM for shoot induction at 25 ± 5°C.

Histochemical GUS Assay

The putative transformed plants were evaluated for the presence of gus expression (transient and stable). For transient gus expression, the treated explants were checked immediately after the infection, and the stable gus expression was analyzed with 3 months old kanamycin resistant regenerated green shoots. The shoots were incubated at 37℃ overnight (in the dark) in 1 mM 5-Bromo-4-chloro-3-indoyl β-D-glucuronide-cyclohexylammonium salt (X-Gluc), 0.1 M sodium phosphate buffer (pH 7.0), 0.1mM potassium ferrocyanide, 0.1 mM potassium ferricyanide, 0.1% Triton X-100. Following incubation, the chlorophyll of the explants was removed using 70% (v/v) ethanol.

Molecular Characterization

Extraction of genomic DNA from young leaf tissues of putatively transformed and control wild plants of *O. sanctum* was done as per the CTAB protocol.[10] Polymerase chain reaction (PCR) amplification was carried out with gene-specific primer for both *npt-II* and *gus-A* gene

[F: 5'ATCAGGATGATCTGGACGAAGAG3' and *npt-II* R:5'CAAGCTCTTCAG CAATATCACG3' and F: 5'ACTGTAACCACGCGTCTGTTGAC3' and *gus-A* R: 5' TGTTTGCCTCCCTGCTGCGG 3']. Amplification was carried out for 5 min at 94°C as denaturation, followed by 32 & 35 cycles of 30 s & 1 min at 94°C as denaturation, then annealing at 56 & 52°C for 30 s, and further final elongation was done at 72°C for 30 s (5 min) & 45 s (10 min), for kanamycin resistant (*npt-II)* and (β-glucuronidase) *gus-A* gene respectively. The amplified PCR products were resolved on 1.2% (w/v) agarose gel.

Statistical Analysis

The experimental set up was designed in a completely randomized manner consisted of 30 explants per petriplate and each treatment/experiment was repeated thrice. All data including shoot/root induction, regeneration, and proliferation were carried out using one-way analysis of variance (ANOVA). Statistical analysis was done using GraphPad Prism 5.0 (GraphPad Sofware. Inc., USA). Values with asterisk are significantly different from W type at $P < 0.05$.

Results and Discussion

Direct Regeneration and Multiple Shoot Induction in *O. sanctum*

To develop a suitable expression system, it is a prerequisite to have efficient regeneration and genetic transformation methods that must be repeatable and easily reproducible. The success of ATMT method and genetic transformation frequency is directly correlated with the regeneration frequency, which provides a pavement to recover multiple micro shoots as well as transgenics. Therefore, an efficient and reproducible protocol is required to achieve the appropriate results.

In order to induce direct regeneration in *O. sanctum*, several explants (leaf, internode, petiole, hypocotyls, etc.) were placed on full strength basal media fortified with a number of combinations of PGRs (Fig. 1(a–o)). Along with the shoot regeneration, formation of embryo like structures was also observed but did not germinate into complete

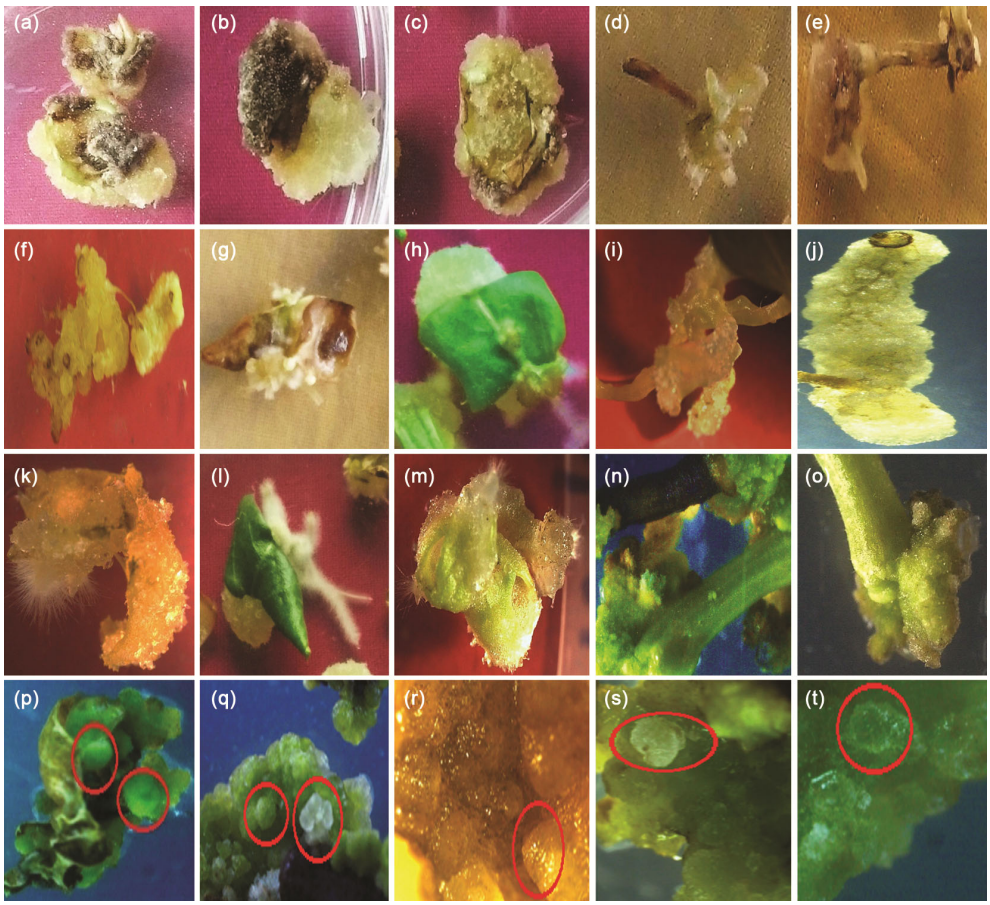

Fig. 1 — Influence of several different combinations of plant growth regulators on various explants for direct regeneration and multiple shoot induction on *O. sanctum*

plantlet (Fig. 1 p-t). However, the direct regeneration and multiple shoot induction/proliferation were successfully achieved maximally through petiole followed by hypocotyl, and leaf explants (Fig. 2(a–c)). In the present study, *de novo* axillary/apical shoot regeneration was achieved successfully from petiole are in concordance with those for *Populus*, *Jatropha*, and pigeon pea.[11–14] *In vitro* petioles were incubated with MS having different concentrations of BAP (cytokinin) and NAA (auxin). The concentration of 3 mg/L BAP and 1 mg/L NAA was found to be most effective among all combinations (Fig. 2(d, e)). The same combination yielded the maximum number (9.6) of regenerated shoots per explant with the highest shoot regeneration rate of 98%. At the same time, further change (either increase or decrease) declines both regeneration rate and quantity of buds originating from single explants (Table 1). The obtained result indicates that the efficiency of *de novo* shoot induction was influenced significantly at different concentrations. However, it has reported that an exogenous supply of cytokinins (with or without auxin) stimulates axillary shoot formation *in planta*.[15] Although in the present study, when BAP and NAA concentration increased from 1 to 3 mg/L BAP and 0.5 to 1 mg/L NAA, significant variation in regeneration percentage of regenerated axillary shoots was observed. Similarly, the number of induced apical shoots per explant was also affected accordingly. On further increase in hormonal/PGR concentration, the regenerated green shoots became supra-optimal and thus resulted in hyper-hydricity without any further proliferation and regeneration. It strongly agrees with the results obtained during the present study that elevating the cytokinin (BAP) in SIM induced *de novo* apical shoot formation[16–18] but at the same time, reduced cytokinin resulted in less number of

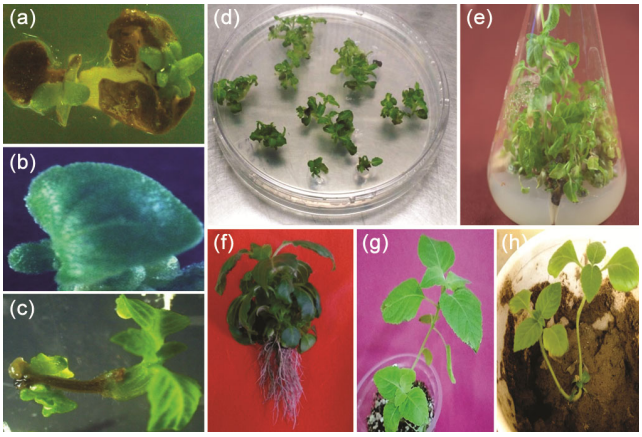

Fig. 2 — Direct regeneration, Multiple shoot proliferation, and Root induction in *O. Sanctum*: Shoot regeneration from (a) hypocotyl, (b) cotyledonary leaf, (c) petiole, (d) Petioles showing multiple shoot induction, (e) *In-vitro* culture showing multiple shoots, (f) *In vitro* regenerated plantlets with healthy root system (g, h) Hardening and acclimatization

Table 1 — Effect of Benzylamino purine (BAP) and Naphthalene acetic acid (NAA) on direct shoot induction and proliferation response using different explants of *O. sanctum*

Media Used (MS+BAP+NAA) mg/L	Explant					
	Hypocotyls		Cotyledonary Leaf		Petioles	
	Shoots/explant	Response (%)	Shoots/explant	Response (%)	Shoots/explant	Response (%)
0.5 + 0.5	1 ± 0.7	10.96 ± 0.85	1.033 ± 0.95	7.36 ± 0.27	—	—
0.8 + 0.5	1.61 ± 0.81	9.16 ± 0.87	1.47 ± 0.49	6.70 ± 0.29	—	—
1 + 0.5	1.13 ± 0.32	9.6 ± 0.92	2.0 ± 0	10.7 ± 0.65	3.66 ± 0.28	30 ± 1.00
1.5 + 0.5	0.5 ± 0.3	13.63 ±1.01	1.28 ± 0.62	6.3 ± 0.33	5.0 ± 1.00	46.6 ± 0.57
2 + 0.5	0.43 ± 0.15	15.8 ± 0.50	1.68 ± 0.60	4.46 ± 0.43	7.6 ± 1.52	73.3 ± 2.08
2.5 + 0.5	0.86 ± 0.41	9.43 ± 0.91	2.17 ± 0.18	7.16 ± 0.41	7.0 ± 1.73	55 ± 1.15
3 + 0.5	1.06 ± 0.25	6.33 ±1.09	1.13 ± 0.28	1.8 ± 0.31	8.33 ± 0.57	63.3 ± 1.15
1 + 1	1.18 ± 0.16	9.48 ± 0.46	2.08 ± 0.86	4.28 ± 0.02	2.60 ± 1.52	15 ± 0.50
1.5 + 1	1.32 ± 0.58	8 ± 2.06	0.91 ± 0.13	2.20 ± 0.29	1.60 ± 0.28	18 ± 0.51
2+1	2.46 ± 0.80	3.33 ± 0.327	2.82 ± 0.28	1.87 ± 0.22	2.70 ± 1.15	20 ± 0.76
2.5+1	2.16 ± 0.22	3.73 ± 0.380	2.22 ± 1.30	4.37 ± 0.66	7.33 ± 2.08	70 ± 1
3 + 1	1.58 ± 0.52	5.76 ± 0.80	1.66 ± 0.57	2.63 ± 0.28	9.67 ± 0.57	98.3 ± 0.28
1 + 1.5	0.94 ± 0.37	3.3 ± 0.38	1.10 ± 0.83	3.40 ± 0.30	—	—
1.5 + 1.5	0.89 ± 0.21	3.33 ± 0.57	1.25 ± 0.69	7.35 ± 0.64	—	—
2 + 1.5	1.81 ± 1.27	4.83 ± 0.50	2.19 ± 1.32	9.50 ± 0.89	2.30 ± 2.08	53.3 ± 0.28
2.5 + 1.5	1.35 ± 0.90	6.63 ± 0.57	3.53 ± 2.49	3.96 ± 0.26	5.30 ± 2.09	70 ± 1.0
3 + 1.5	1.64 ± 0.51	9.56 ± 0.93	2.34 ± 1.11	3.06 ± 0.55	8.4 ± 1.15	73.33 ± 1.15

regenerated shoot (Table 1).[19] Additionally, the high cytokinin concentration inhibited the induction and proliferation of shoots and hence several symptoms such as rosette shoots and vitrification were also recorded. [20,21]

Therefore, the present study highly corroborates to previous findings, in which the existence of crosstalk between auxin and cytokinin signaling showed to interact both synergistically and antagonistically during the development of axillary meristems.[22–26] Furthermore, the results achieved were in line with previous reports that showed significant achievements in terms of direct regeneration.[7,11,27] *De novo* plant regeneration from petiole was achieved successfully through synergistic interaction of hormones (cytokine and auxin) directly.[28,29] An efficient and highly reproducible method for continuous high-frequency shoot induction and multiplication along with plantlets production in *O. sanctum* was developed. The present study could be helpful in studying the crosstalk between hormonal-induced and endogenous programs that have not well understood yet. Also, the protocol is highly proficient and reproducible to induce large scale clonal and micro propagation in *O. sanctum.*

Root induction of *in vitro* regenerated shoots

Green regenerated microshoots (1–1.5 cm) were transferred to full and half-strength MS medium, along with MS having varied concentrations of IBA or NAA (0.1–1.5 mg/L) for root induction. After 4–5 weeks of culture, it was observed that half-strength MS basal medium significantly influenced the response and frequency of root induction than that of full strength basal MS along with MS fortified with either IBA or NAA as shown in Table 2. Results showed that ½ MS basal medium could effectively stimulate the initiation and growth of roots, inducing the best effect and the highest root induction rate (85 ± 0.35%). IBA and NAA were found to be efficient in the root induction *in planta.*[30] However, in our study, supplementing with either IBA or NAA had not shown any significant result. The results obtained are in conformity with the previous results[31] (Table 2). The roots regenerated on 1/2 medium were healthy, while those on full-strength MS medium and IBA /NAA were tender, slim, and eventually turned to black. The root formation was initiated after 2–3 weeks of subculture, and the regenerated plantlets were observed to have well-established root system (Fig. 2f). These plantlets were further transferred and hardened into plastic pots filled with equivalent ratio of vermiculite and sand for acclimatization and further growth (Fig. 2(g, h)).

Table 2 — Influence of NAA or IBA, half and full strength MS on rooting of *in vtiro* derived shoots of *O. sanctum*

Media composition	Roots/explants	Response (%)
Half strength MS	7.6 ± 0.27	85 ± 0.35
Full strength MS	4.4 ± 0.3	60 ± 1.5
MS + 0.1 NAA	4.0 ± 0.49	40 ± 3.44
MS + 0.5 NAA	4.46 ± 0.38	40 ± 2.08
MS + 1 NAA	4.1 ± 0.25	43 ± 1.20
MS + 1.5 NAA	3.5 ± 0.17	37 ± 0.8
MS + 0.1 IBA	3.7 ± 0.24	45 ± 0.17
MS + 0.5 IBA	3.2 ± 0.1	58 ± 0.15
MS + 1 IBA	3.28 ± 0.31	42 ± 0.23
MS + 1.5 IBA	2.4 ± 0.29	48 ± 0.06

Agrobacterium Tumefaciens Mediated Genetic Transformation Studies in *O. sanctum*

Genetic transformation in *O. sanctum* was done, using LBA4404 strain of *A. tumefaciens* harboring pBI121 as a binary vector (Fig. 3a). To achieve the maximum transformation effectiveness, several parameters influencing the transformation frequency were optimized prior to the ATMT experiment. It includes optimization and standardization of effective dose of kanamycin, infection time, the concentration of AS, and use of tween-20.

The sensitivity of the explants to kanamycin was assessed by adding varying concentrations of kanamycin (10–60 mg/L) in SIM and visually checking the regeneration of explants after 3–4 weeks of culture. The petioles cultured on MS medium without kanamycin (0 mg/L) were healthy, and green showed the highest regeneration responses. The use of *npt-II* as a selection marker and desired gene integrated within the same vector provides an efficient and convenient screening system. In the present study, explants at 30 mg/L kanamycin showed a regeneration frequency of 70% ± 0.76 with healthy green shoots. However, on further increase (40 mg/L); the regeneration frequency was declined (13% ± 0.28). Meanwhile, the untransformed regenerated shoots inoculated on 50–60 mg/L kanamycin have shown complete bleaching along with the induction of albino microshoots that eventually turned to brown (Fig. 3b). The result can be correlated with previous findings where kanamycin was used as a successful screening marker.[32] Therefore, for present study, 50 mg/L of kanamycin concentration was optimum as the minimum inhibitory concentration to remove pseudo-escapes and effective screening of putative transformants.

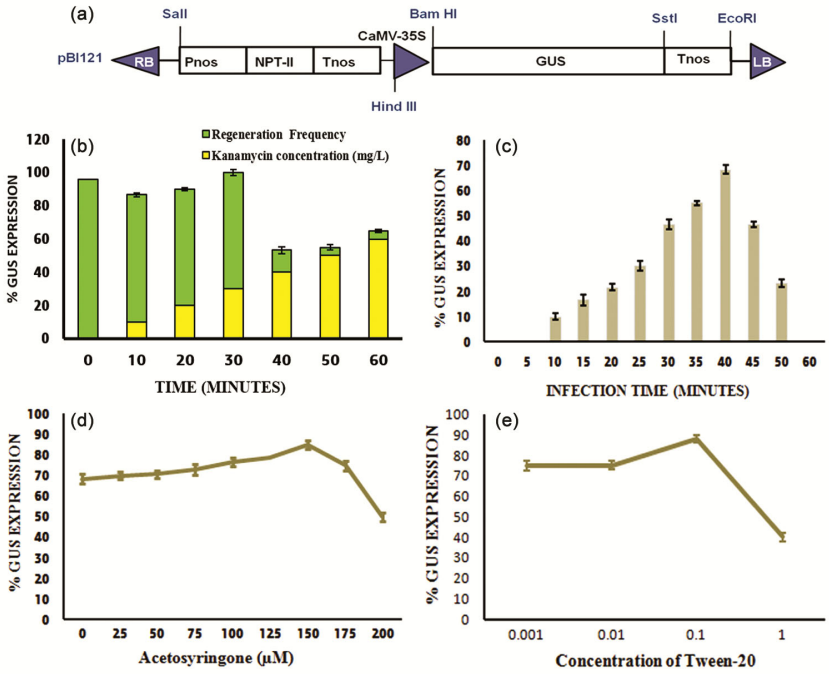

Fig. 3 — T-DNA construct map and optimized parameter for *A. tumefaciens* mediated genetic transformation in *O. Sanctum*: (a) Binary vector pBI121 with restriction sites and *gusA* with intron; and Effect of (b) Kanamycin, (c) Infection time, (d) Acetosyringone, and (e) Surfactant (Tween-20); on shoot regeneration frequency of transgenic *O. sanctum*

The *Agrobacterium* concentration was previously shown to affect transformation frequencies in many genetic transformation studies. During the present study, *Agrobacterium* suspension of OD600 (0.6) concentration was used to infect the petiole explant. The optimum treatment time for achieving maximum transient gus expression frequency (68.3% ± 0.76) was observed to be 40 min (Fig. 3c). It was found that petioles dipped/treated for 10–30 min in bacterial solution was not optimum as the transient gus expression was very low. However, on further increase in infection time, the gus expression rate was significantly declined (20%) resulted in browning and necrosis of explants. Additionally, it has also been observed that the concentration of AS supplied during transformation study is also a crucial parameter. The established gus transient expression was observed to varied along with AS concentration that was supplemented with CCM which stimulates Vir genes expression in Ti-plasmid.[33] There was a considerable enhancement in gus expression frequency with 150 µM AS resulted in the highest transformation efficiency (85% ± 1.32) in *O. sanctum* (Fig. 3d). With further increase in the concentration of AS the transformation event decreases. The varied concentration from 50–200 µM of AS has been used in plant system depending upon the type plant species. The beneficial effect of AS has been used to maximize the transformation frequencies by many workers.[34] Nonetheless, above stated parameters played an important role in establishing an efficient system for ATMT but the use of Tween-20 during transformation study has also been observed to be highly significant. The use of surfactant in many monocots and dicots plants showed significant results in enhancing the transformation efficiency[7] by reducing the surface tension, and enhancing the chances of bacterium to penetrate inside the plant cell. Along with the 150 µM AS and 40 min of infection time, 0.1% (v/v) of tween-20 was found optimum to achieve highest gus expression in *Ocimum sanctum* (Fig. 3e).

Regeneration, Gus expression and Molecular Analysis

The optimized factors for *in vitro* regeneration and genetic transformation method using petioles as explants was proved to be highly efficient in *O. sanctum*. After regeneration, the *in vitro* grown healthy shoots were transferred on the screening medium supplemented with 50 mg/L kanamycin to remove the pseudo-transformants. The regenerated shoots which survived on screening medium were

further (1–1.5 cm) placed onto half-strength MS medium for root initiation. The putatively transformed plantlets with proper root systems were shifted to glasshouse for further growth and development.

Meanwhile, after 4–5 days of inoculation on SIM with kanamycin (50 mg/L), healthy & green tissues were assessed for transient gus expression. The frequency and presence of gus expression was checked through histochemical staining using different parts of the *in vitro* regenerated putative transgenics. Distinct blue-stained cells (spots) were observed in several part of the plant such as midrib, veins of the leaves along with petioles, stem, etc. In *O. sanctum*, the GUS-expressing cells displayed as blue-stained small spots, rather than the large blue pattern, similar to experiments done earlier.[35] The blue-colored cells were first appeared near veins, and eventually evenly distributed on all sides of the leaf explant, rather than preferentially at the basal end. However, stable gus expression was checked after 3 months of inoculation of putative transgenics surviving on 50 mg/L kanamycin screening medium. The results showed 70% ± 0.35 of stable transformation frequency in *O. sanctum*. The presence of expression was analyzed from randomly selected putatively transformed healthy tissues growing as kanamycin resistant shoots. It was observed that the blue color expression was prominent in leaves, shoot tips, stem, inflorescence, and petioles while no gus expression was seen in control tissues (Fig. 4). Afterwards the confirmation was done by extracting the genomic DNA from putatively transformed and

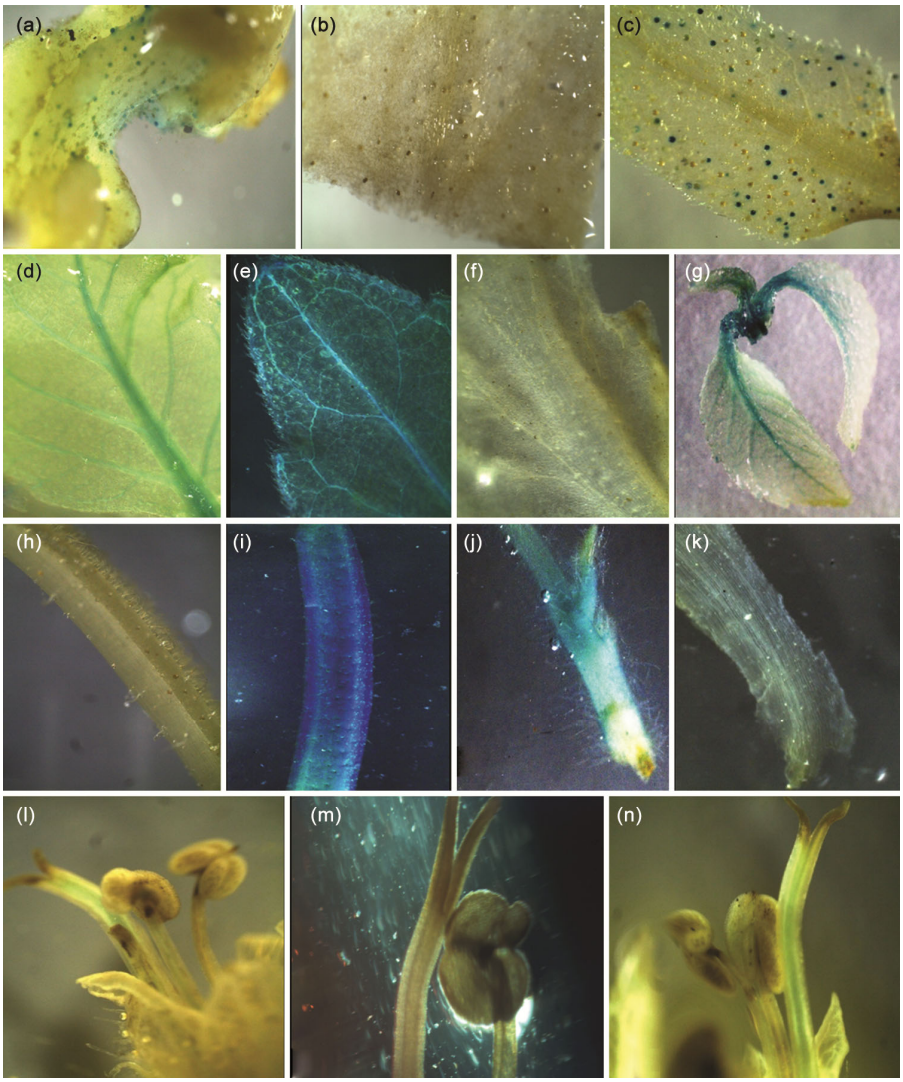

Fig. 4 — Histochemical Gus expression in *O. sanctum* transgenic plants. (a, c) transient gus expression in leaf; (d, e, g) gus expression shown in whole transformed leaf and apical node; (i, j) gus expression in transformed stem and petiole; (l, n) transformed inflorescence and (b, f, h, k, m) non-transformed control explants

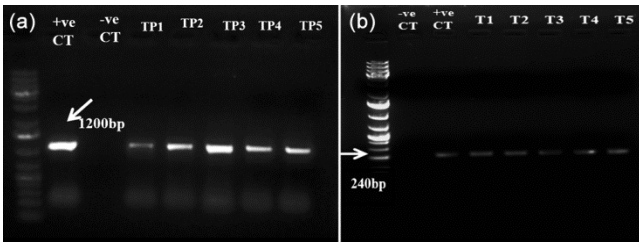

Fig. 5 — Molecular analysis of transgenic *O. sanctum* plants (**a**) PCR amplification of *gus-A* gene (1200 bp), showing; positive control (pBI-121 plasmid), negative control (wild) and TP1-TP5: gus positive transformants (**b**) PCR amplification of *npt-II* gene (240 bp), positive control (pBI-121 plasmid), negative control (wild) and T1-T5: transformants

non-transformed plants and subjected to PCR analysis. All putative transgenics showed positive results with a band corresponding to approximately 1200 bp and 240 bp for *gus-A* and *npt-II* genes, respectively showing the successful integration of *both* genes in *O. sanctum transgenic plants* (Fig. 5).

In gene transfer technology, transient gus expression is highly advantageous because of the factors like speedy in nature, enabling researchers to get results weekly. However, the stable gus expression is highly recommended for its versatility, simplicity, and robustness. Previously many researchers have used transient and stable gus expression as a marker system for assessing the functionality, localization, and transferring of the novel genes. Therefore, in the present study, for the first time, we are describing transient as well as stable gus expression system in *O. sanctum* (*O. tenuiflorum*). In contrast to the previous studies regarding established genetic protocols in *O. basilicum* and *O. citriodorum*; where only transient expression was studied while comparing two different *Agrobacterium* strains GV3101 and EHA105.[8] Similarly, in *O. gratissimum*, only 20% ± 0.7 of stable transformation frequency was achieved.[7] Furthermore, the established protocol showed 70% ± 0.35 gus expression frequency. Therefore, the technique is highly efficient and reliable in addition to the fact that there has no ATMT protocol been reported till date in *O. sanctum L.*

Conclusions

Ocimum sanctum (*O. tenuiliflorum or 'cim-ayu'*) being rich in a variety of bioactive phyto-constituents including, polyphenols, flavanols, triterpenes, saponins etc. that may have a significant biological activity shows high difficulty to establish a prominent and reproducible ATMT method. The various factors namely infection time, acetosyringone concentration, use of surfactant etc. showed a great impact on transformation frequency. The optimized factors for *in vitro* regeneration *i.e*. modified MS medium containing BAP & NAA in the ratio of 3 & 1 mg/L respectively and genetic transformation protocol [40 min of treatment with $OD_{(600)}$, 0.6 of infection medium, in addition to 150 µM acetosyringone, and 0.1% (v/v) surfactant, and subsequently the treated explants were co-cultivated for 48 h in the dark] resulted in achieving maximum transformation frequency of 70% ± 0.35 in *O. sanctum.*

The established method is a highly proficient *in vitro* protocol for direct regeneration and multiple shoot induction using petiole explant. For the first time, genetic transformation (ATMT) protocol in *O. sanctum* has been developed. The protocol can be used for a wide range of genetic and proteomic manipulations and successful integration of desired gene. The technology/protocol provides a powerful tool to modulate the metabolic flux as well as pathway engineering in order to add high pharmaceutical value *in planta.*

Acknowledgements

All authors wish to express their sincere gratitude to the Director, CSIR-CIMAP, for providing the facilities to carry out this research. The work was supported by grant from CSIR-CIMAP Network Project BSC-203 (CHEMBIO). We acknowledge 'Council of Scientific and Industrial Research (CSIR, New Delhi, India)' for financial support through CSIR-SRF. The CSIR-CIMAP manuscript number is 2020/JUN/51.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1 Darrah H H, *The cultivated basils*, *Independence* (MO Buckeye Printing Company) 1980.
2 Gupta S K, Prakash J & Srivastava S, Validation of traditional claim of Tulsi, *Ocimum sanctum* Linn. as a medicinal plant, *Indian J Exp Biol*, **40** (2002) 765–73.
3 Singh N & Hoette Y, Tulsi: The mother medicine of nature, Lucknow, India: (International Institute of Herbal Medicine) 2002.
4 Uma Devi P, Radioprotective, anticarcinogenic and antioxidant properties of the Indian holy basil, *Ocimum sanctum* (Tulasi), *Indian J Exp Biol*, **39** (2001) 185–190.

5 Shishodia S, Majumdar S, Banerjee S & Aggarwal B B, Urosolic acid inhibits nuclear factor-kappaB activation induced by carcinogenic agents through suppression of Ikappa Balpha kinase and p65 phosphorylation: Correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1, *Cancer Res*, **63** (2003) 4375–83.

6 Pattanayak P, Behera P, Das D & Panda S K, *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: An overview, *Pharmacogn Rev*, **4** (2010) 95–105.

7 Khan S, Fahim N, Singh P & Rahman L, *Agrobacterium tumefaciens* mediated genetic transformation of *Ocimum gratissimum*: A medicinally important crop, *Ind Crops Prod*, **71** (2015) 138–146.

8 Deschamps C & Simon J E, *Agrobacterium tumefaciens*-mediated transformation of *Ocimum basilicum* and *O. citriodorum*, *Plant Cell Rep*, **21** (2002) 359–364.

9 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, **15** (1962) 473–497.

10 Khanuja S P S, Shasany A K, Darokar M P & Kumar S, Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils, *Plant Mol Biol Rep*, (1999) 1–7

11 Huang D & Dai W, Direct regeneration from in vitro leaf and petiole tissues of *Populus tremula 'Erecta'*, *PCTOC*, **107** (2011) 69–174

12 Gawali P M, Akhare A A & Gahukar S J, *In vitro* regeneration of pigeonpea from leaf with petiole explants, *Asian Sciences*, **5** (2010) 34–38.

13 Kumar N & Reddy M P, Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant, *Ann Appl Biol*, **156** (2010) 367–375.

14 Kumar N, Anand K G V & Reddy M P, In vitro regeneration from petiole explants of non-toxic *Jatropha curcas*, *Ind Crops Prod*, **33** (2011) 146–151.

15 Bernula D, Benko P, Kaszler N, Domonkos I, Valkai I, Szollosi R, Ferenc G, Ayaydin F, Feher A, Gemes K, Timely removal of exogenous cytokinin and the prevention of auxin transport from the shoot to the root affect the regeneration potential of *Arabidopsis* roots, *PCTOC*, **140** (2020) 327–339.

16 Catterou M, Dubois F, Smets R, Vaniet S & Kichey T, Onckelen H V, Noreel B S S, Sangwan R S, hoc: an Arabidopsis mutant overproducing cytokinins and expressing high in vitro organogenic capacity, *Plant J*, **30** (2002) 273–287.

17 Kakimoto T, Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP Isopentenyltransferases, *Plant Cell Physiol*, **42** (2001) 677–685.

18 Sun J, Niu Q W, Tarkowski P, Zheng B, Tarkowska D & Sandberg G, Chua N H, Zuo J, The Arabidopsis AtIPT8/PGA22 gene encodes an isopentenyl transferase that is involved in de novo cytokinin biosynthesis, *Plant Physiol*, **131** (2003) 167–176.

19 Cheng Z J, Wang L & Sun W, Zhang Y, Zhou C, Su Y H, LI W, Sun T T, Zhao X Y, Li X G, Cheng Y, Zhao Y, Xie Q, Zhang S X, Pattern of auxin and cytokinin responses for shoot meristem induction results from the regulation of cytokinin biosynthesis by auxin response factor3, *Plant Physiol,* **161** (2013) 240–251.

20 Confalonieri M, Balestrazzi A, Bisoffi S & Carbonera D, *In vitro* culture and genetic engineering of Populus spp.: synergy for forest tree improvement, *PCTOC*, **72** (2003) 109–138.

21 Magyar-Tábori K, Dobránszki J, Teixeira da Silva J A, Bulley S M & Hudák I, The role of cytokinins in shoot organogenesis in apple, *PCTOC*, **101** (2010) 251–267

22 Kurepa J, Shull T E & Smalle J A, Antagonistic activity of auxin and cytokinin in shoot and root organs, *Plant Direct*, 3(2) (2019) 1–9.

23 Besnard F, Refahi Y, Morin V, Marteaux B, Brunoud G, Chambrier P & Vernoux T, Cytokinin signalling inhibitory fields provide robustness to *phyllotaxis*, *Nature*, **505** (2014) 417–421.

24 Chickarmane V S, Gordon S P, Tarr P T, Heisler M G, & Meyerowitz E M, Cytokinin signaling as a positional cue for patterning the apical-basal axis of the growing Arabidopsis shoot meristem, *Proc Natl Acad Sci U.S.A*, **109** (2012) 4002–4007.

25 Dello Ioio R, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita M T, & Sabatini S, A genetic framework for the control of cell division and differentiation in the root meristem, *Science*, **322** (2008) 1380–1384.

26 Truskina J & Vernoux T, The growth of a stable stationary structure: Coordinating cell behavior and patterning at the shoot apical meristem, *Curr Opin Plant Biol*, **41** (2018) 83–88.

27 Chisti N, Shawl A S, Sultan P, Kaloo Z A & Khan F A, *In vitro* organogenesis in *Lavendula officinalis*, a multipurpose plant of industrial importance, *Proc Natl Semin*, 2003, 14

28 Ikeuchi M, Sugimoto K & Iwase A, Plant callus: mechanisms of induction and repression, *Plant Cell*, **25** (2013) 3159–3173

29 Bustillo-Avendaño E, Ibáñez S, Sanz O, Barros J A S, Gude I, Perianez-Rodriguez J, Micol J L, Pozo J C D, Moreno-Risueno M A & Pérez-Pérez J M, Regulation of hormonal control, cell reprogramming, and patterning during DeNovo root organogenesis, *Plant Physiol*, **176** (2018) 1709–1727

30 Rahdari P, Khosroabadi M, Delfani K & Hoseini S M, Effect of different concentration of plant hormones (IBA and NAA) on rooting and growth factors in root and stem cuttings of

Cordyline terminalis, *J Med Bioeng*, **3** (2014) 190–194.

31 Liu Y, Lu J, Zhu H, Li L, Shi Y & Yin X, Efficient culture protocol for plant regeneration from cotyledonary petiole explants of *Jatropha curcas* L., *Biotechnol Biotechnol Equip*, **30** (2016) 907–914.

32 Saha P, Datta K, Majumder S, Sarkar C, China S P, Sarkar S N, Sarkar D & Datta S K, *Agrobacterium* mediated genetic transformation of commercial jute cultivar *Corchorus capsularis* cv: JRC 321 using shoot tip explants, *PCTOC*, **118** (2014) 313–326

33 Jin S, Zhang X, Liang S, Nie Y, Guo X & Huang C, Factors affecting transformation efficiency of embryogenic callus of Upland cotton (*Gossypium hirsutum*) with *Agrobacterium tumefaciens, PCTOC,* **81** (2005) 229–237

34 Sheikholeslam S N & Weeks D P, Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*, *Plant Mol Biol*, **8** (1987) 291–298

35 Janssen B & Gardner R C, Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*, *Plant Mol Biol*, **14** (1989) 61–72