
Research Article

Efficient *Agrobacterium* mediated genetic transformation of CRISPR/Cas9 construct using cotyledonary node explants of greengram

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Abstract

An efficient protocol for generating transformed greengram plants by choosing prominent explant which is used to generate transformed plantlets for their survivability and also with high transformation efficiency. However, the crop production is reduced due to factors. In current work, we employed transgenic free approach using CRISPR/Cas9 tool for establishing transformation protocol. The CRISPR/Cas9 is getting momentum widely for developing transgene free crops. In this approach, guide RNA encoding for ~18-23 bp sequence that contains a PAM (protospacer adjacent motif) on either strand of DNA, where CRISPR incepts with a single guide RNA (sgRNA) to target genes and initiate excision of a complimentary strands through the Cas9 endonuclease. Explants' effects on CRISPR/Cas9 construct transformation were studied. For transformation, three distinct explants cotyledonary node (CN- MSB₅ +3% sucrose+ 0.5 mg/L BAP for CN), shoot tip (ST-MSB₅ + 3% sucrose+ + 0.5 mg/L BAP + 0.01 mg/L NAA), and immature cotyledon (IMC-3% sucrose + 0.5 mg/L BAP + 0.1 mg/L NAA) were used. The prepared explants were infected with EHA105 strain of *Agrobacterium tumefaciens* harboring a binary vector of pMDC100 containing *npt II* gene as screenable marker and Cas9. The explants were selected on 50 mg/L kanamycin medium. The transformed plants were confirmed by PCR using *npt II* and Cas9 specific primers. Cotyledonary node explant was found to be more efficient for CRISPR/Cas9 transformation and also it promisingly showed transgene integration through PCR analysis having more transformation efficiency and can be used for the production of transgenic free greengram crop using CRISPR/Cas9 approach.

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1. Introduction

Mungbean (*Vigna radiata* L) is an essential source of protein, originated in India about 3500 years ago and spread to other countries like USA, Canada, Australia etc. (Nair, 2012). Mungbean commonly named as greengram, being cultivated in an area of 7.3 million hectares with an average yield of 721 kg/h, procuring 5.2 million tons globally (Nair and Schreinemachers 2020). However, greengram production is subsided due to its susceptibility to numerous diseases including yellow mosaic disease (YMD), Cercospora leaf spot, powdery mildew, root rot and with various insect pests like aphids, pod borers, whiteflies, stink bugs and bruchids (Borah and Dasgupta 2012). In addition to the aforementioned reasons, other factors that contribute to the lesser generation of greengram include: (i) reduced genetic diversity, (ii) changes in the yield owed as a result of severe environmental stress, such as salinity drought and photoperiod restriction (iii) poor cultural usefulness (iv) reduced pod development owing to synchrony, and (v) high incidence of insect infestation are further factors (Parihar et al. 2017; Chand et al. 2015; Gnanaraj et al. 2015). Greengram cultivars currently lack the qualities necessary to combat against invasive pests, uncontrolled illnesses, and periodic irregularities. Due to insufficient amounts of the desired gene(s) in the germplasm, extensive self-pollination, and cross-animosity incompatibility between wild and produced types, traditional breeding has limited effectiveness.

Conventional breeding is very intensive as it takes about 8-12 years due to non availability of desirable genes. Gardner et al. (1993) used an approach to introduce desired genes, which are in accessible *via* conventional breeding methods. Genetic engineering is an excellent tool for developing new characters into plant without altering its existing traits. A reliable genetic transformation is an effective method to insert genes of interest in mungbean cultivars which are absent in current genotypes, leading to improve its production by transgenic approach. Recent studies like CRISPR based genome editing and its related transformation studies through *in-vitro* regeneration, providing a protocol by which a cell or tissue can regenerate into whole plant. In pulses, many tissue culture protocols were generated so far, greengram (mungbean) (Yadav et al., 2012, 2010), urdbean (Adlinge et al., 2014) and cowpea (Tie et al., 2013). Greengram regeneration protocols were developed via organogenesis process (Himabindu et al. 2014) and somatic embryogenesis (Sivakumar et al. 2010). Regeneration in greengram is standardized by using three explants *i.e.*, cotyledonary node (CN), shoot tip (ST), and immature cotyledon (IMC), however to generate high transformation efficiency, optimization with modified process of consistent transformation protocol is needed.

Despite the availability of several protocols on regeneration, greengram being highly recalcitrant crop, the explant variability for transformation is a major problem. Hence, it is necessary to optimize greengram regeneration protocol for the generation of clean and transgene free varieties. In this regard CRISPR/Cas9 would cater the consumer friendly crop production for developing resistant crop plants by emerging a generation which significantly enhance new plant along with various traits in the germplasm. Shoot tip survivability at selection stage is problematic, whereas callus mediated *via* immature cotyledons is not reliable due to lack of fibrous, embryogenic calli for transformed efficiency. In this present study, three different explants such as ST, CN and IMC were infected with *Agrobacterium* harbouring CRISPR/Cas9 construct. Among the three tested explants, cotyledonary nodes were found to be promisingly showed transgene integration through PCR analysis having more transformation efficiency and it is further chosen as a starting material for transformation with CRISPR/Cas9 construct.

2. Materials and methods

2.1 Plant source and cultural conditions

Seeds of LGG460 genotype of greengram collected from RARS, Lam Farm, ANGRAU, and Guntur. Mature seeds were sterilized three times with sterile distilled water, then treated them with fungicide (1 % (w/v) Bavistin) for about 10 min and again they were surface sterilized with 70 %

ethanol for 30 sec and by subsequent washes by 0.1 % (w/v) Mercuric chloride (HgCl₂) for 5 min. Seeds were thoroughly cleaned further with sterile water for 4-5 times. About 15 sterilized seeds were blotted on sterile filter paper and inoculated on MSB₅ basal medium with 3% sucrose + 0.8 % agar without hormone supplement. After 2-3 days of germination the seeds were excised for the preparation of CN, ST and IMC explants. The prepared explants were used for *in-vitro* regeneration and the established regeneration system was used to generate the transgenic green gram plants.

2.2 *Agrobacterium tumefaciens* culture and vector construction

EHA105 strain of *Agrobacterium tumefaciens* harbouring plasmid pMDC100 was used for transformation with three different explants *i.e.*, CN, ST and IMC. The binary vector pMDC100 contains a screenable marker gene *i.e.*, *npt II* resistance to kanamycin along with gRNAs *AtU6p-AC1-AtU6t*, *AtU3p-AV1-AtU3t* and Cas9 (*DE35S-Cas9-Nos-t*) cassette which was electroporated into *A. tumefaciens* (EHA105). For *Agrobacterium* growth 25 ml of YEM broth (Hi-media) containing 50 mg/L each kanamycin, streptomycin and rifampicin, as well as 20 mg/L was inoculated with newly grown *Agrobacterium* culture incubated at 28 °C at 180 rpm for 16 hrs. After centrifuging the culture for 10 min at room temperature for 5000 rpm the pellet was resuspended in infection medium with an OD₆₀₀ of 0.5 for the infection of explants.

2.3 Explants preparation and Co-cultivation with *Agrobacterium*

Cotyledonary node (CN): CN explants were excised from 2-day old seedling grown in MSB₅ media supplemented with 3% sucrose along with 2 mg/L Benzylaminopurine (BAP) + 0.8 % agar. CN was pricked with syringe needle at axillary meristematic region for co-cultivation with *Agrobacterium* culture that was carrying with CRISPR Construct. The explants with culture were kept in incubator and agitated for 10 min at 28 °C. The culture after infection was then decanted and explants were blot dried on sterile filter paper. Co-cultivated plates were maintained at 25°C for two days under dark in co-cultivation media (MSB₅ + 3% sucrose +0.5 mg/l Benzylaminopurine (BAP) + 200 µM acetosyringone + 0.8% agar). Explants from healthy controls were also cultivated in comparable circumstances. Following co-cultivation, explants were five times washed with 250 mg/L cefotaxime, dried on sterile filter paper, and then placed the explants on elongation medium for shoot elongation (MSB₅ + 3% sucrose + 0.5 mg/L Benzylaminopurine (BAP) + 0.8% agar) with addition of 50 mg/L kanamycin and 250 mg/L cefotaxime. These explants were grown for 30 days with two selection cycles separated by 15 days on selection media comprising MSB₅ +3% sucrose + 0.5 mg/L Benzylaminopurine (BAP) + 250 mg/L Cefotaxime + 50 mg/L Kanamycin + 0.8% agar. The regenerated shoots were transferred to sterile moist cotton mixed with liquid MSB₅ 1/2 (half strength).

Shoot tip (ST): ST explants were excised from 3 day old aseptically grown seedling of LGG460 genotype inoculated on MSB₅ basal medium. Two cotyledons attached at the CN is removed by cutting the seedling at 2 mm below the nodal region along the middle of the seed leading to production of shoot tip explant. Shoot tip region were pin-pricked with fine needle and co-cultivated with *Agrobacterium* culture harbouring with pMDC100. The infected explants were blot dried on sterile filter paper after being incubated with the culture for 5 minutes at 28 °C. Agro-infected explants were strengthened by culturing in co-cultivation media (MSB₅ + 3% sucrose + 0.5 mg/l Benzylaminopurine (BAP) + 200 µM acetosyringone + 0.8% agar) and nurtured at 25 °C for 2 days under dark. Also cultivated under same circumstances were uninfected control explants. Cefotaxime is used in a manner similar to CN for cleaning the co-cultivated explants. These explants were grown for 30 days on selection media containing MSB₅ + 0.5 mg/L Benzylaminopurine (BAP) + 0.01 mg/L NAA + 250 mg/L Cefotaxime + 50 mg/L Kanamycin + 0.8% agar, with two selections of 15 d each. Regenerated shoots were transferred to culture tube with sterile cotton that has been moistened with ½ (half) strength MSB₅+1 mg/L IBA liquid medium for effective rooting for plant strengthening.

2.4 Immature cotyledons for callus mediated transformation:

IMC were collected from immature pods. The young cotyledons were pin-pricked with fine needle and incubated with *Agrobacterium* culture harbouring pMDC100 with incubation of 5 min. The explants were kept for drying by blotting. Following which explants were co-cultivated on callus induction medium (MSB₅ + 0.5 mg/L Benzylaminopurine (BAP) + 0.1 mg/L NAA + 200 µM acetosyringone + 0.8 % agar) and incubated for two days. The explants were washed similar to procedure followed for the CN. These explants were cultured on selection media containing MSB₅ + 0.5 mg/L Benzylaminopurine (BAP) + 0.1 mg/L NAA+ 250 mg/L Cefotaxime + 50 mg/L Kanamycin+ 0.8 % agar) for 30 days two selection cycles with 15 days interval until fibrous calli formation was observed. Regenerated calli was transformed into regeneration medium with MSB₅ + 0.5 mg/L Benzylaminopurine (BAP) + 250 mg/L Cefotaxime for 15 days to multiple from the calli. For effective root development, the regenerated shoots were placed in a culture tube with wet cotton supplemented with liquid half strength MSB₅+ 1 mg/L IBA medium.

Table 1. The greengram explants at different stages of transformation by using concentrations of Auxins and cytokinins

S.No	Greengram explants	Germination	Cocultivation	Selections	Regeneration	Rooting
1	Cotyledonary node	MSB ₅ +3% sucrose+2 mg/L BAP	MSB ₅ +3% sucrose+0.5 mg/L BAP +200µM acetosyringone	MSB ₅ +3% sucrose+0.5 mg/L BAP +50 mg/L kanamycin +250 mg/L Cefotaxime	MSB ₅ +3% sucrose+0.5 mg/L BAP +50 mg/L kanamycin	1/2 MSB ₅ + 1 mg/L IBA (Liq)
2	Shoot tip	1/2 MSB ₅	MSB ₅ +3% sucrose+0.5 mg/L BAP +200µM acetosyringone	MSB ₅ +0.5 mg/L 3% sucrose+ BAP + 0.01 mg/L NAA +50 mg/L kanamycin +250 mg/L Cefotaxime	MSB ₅ +3% sucrose+0.5 mg/L BAP + 0.01 mg/L NAA +50 mg/L kanamycin	1/2 MSB ₅ + 1 mg/L IBA (Liq)
3	Immature cotyledons		MSB ₅ +3% sucrose+0.5 mg/L BAP+0.1 mg/L NAA+200µM acetosyringone	MSB ₅ +3% sucrose+0.5 mg/L BAP+0.1 mg/L NAA+50 mg/L kanamycin +250 mg/L Cefotaxime	MSB ₅ +3% sucrose+0.5 mg/L BAP+0.1 mg/L NAA+50 mg/L kanamycin	1/2 MSB ₅ + 1 mg/L IBA (Liq)

2.5 Acclimatization

After 15 days, mature plantlets were moved vermiculite-filled plastic cups with clear plastic and kept for hardening in a controlled greenhouse environment. The transgenic glasshouse was used to grow the plants till their maturity.

2.6 Molecular characterization of putative transgenics

PCR amplification was carried out with *npt II* and *Cas9* genes using specific primers and genomic DNA (Vanti et al., 2015) from putative T₀ transgenic plants, untransformed plants (negative control) and pMDC100 plasmid (positive control) as templates. A PCR reaction volume of 10µl

containing 2 μ L *Taq* buffer (10X), dNTPs of working stock of 2.5mM, 10 pmol of forward and reverse primer, 5 U of *Taq*, 100 ng of plant genomic DNA was used as a template from transformed and control plants. For the *nptII* gene the primers used were: forward 5'ACTCGTCAAGAAGGCGA-TAGAAG 3' and reverse 5'TCTCCGATAAGCCGATACTGAC 3' whereas, the primers for the Cas9 gene were: forward 5'CGAAGAGGAACAGCGACAAG 3' and reverse 5'GAGGGTGAAGAGATGGATGATG 3'. The reaction mixture underwent 35 cycles i.e., denaturation for 40 s at 94°C, annealing temperature of *Cas9* gene at 54 °C and *npt II* gene at 57 °C for 40 s, followed by initial extension for 40 s at 72 °C and final extension of 7 min at 72 °C in a thermal cycler (ABI system). The amplified PCR products of *Cas9* and *npt II* were gel run with 1% agarose and visualized in gel doc system (G-Box Syngene, UK) (Figure 5).

3. Results and discussion

3.1 Multiple shoot induction by *Agrobacterium* mediated transformation by regeneration

The pMDC100 vector harbouring *AtU6p-AC1-AtU6t*, *AtU3p-AV1-AtU3t* and *Cas9* cassettes was mobilized into *Agrobacterium* EHA105. The purpose of inducing numerous shoots, various types of explants such as CN, ST and IMC were examined on MS and B5 media supplemented with different concentrations alone or in combination with NAA for multiple shoot induction. The impact of different plant hormones concentration is presented in Table 1.

Agrobacterium (EHA105) harboring pMDC100-CRISPR construct was used to infect two batches of greengram (LGG-460) CN explants (~60/ per batch), ST explants (~60/ per batch) and IMC (~60/ per batch). A total of 120 explants from each explant revealed resistance to kanamycin (50 mg/L) in 32 of (CN), 28 of (ST), and 15 of (IMC) of them respectively, (Figure 1, Figure 2, and Figure 3).

The maximum number of multiple shoots were formed in the media containing of MSB₅ + 0.5 mg/L BAP for CN, MSB₅ + 0.5 mg/L BAP + 0.01 mg/L NAA for ST and MSB₅ + 0.5 mg/L BAP + 0.1 mg/L NAA for IMC explants (Figure 1, Figure 2, and Figure 3).

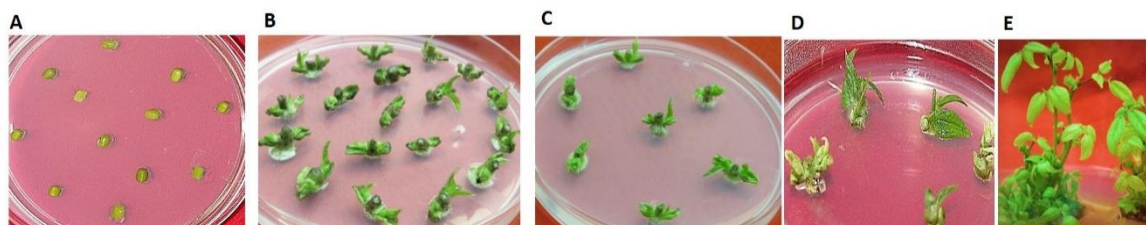


Figure 1. *Agrobacterium*-mediated transformation of greengram Cotyledonary node as explants with CRISPR construct: A. Inoculated seeds in MSB5 medium B. Co-cultivated cotyledonary node explants with the CRISPR construct C. explants inoculated on kanamycin media for selection D. kanamycin resistant explants on regeneration medium E. explants retrieved were grown on elongation medium

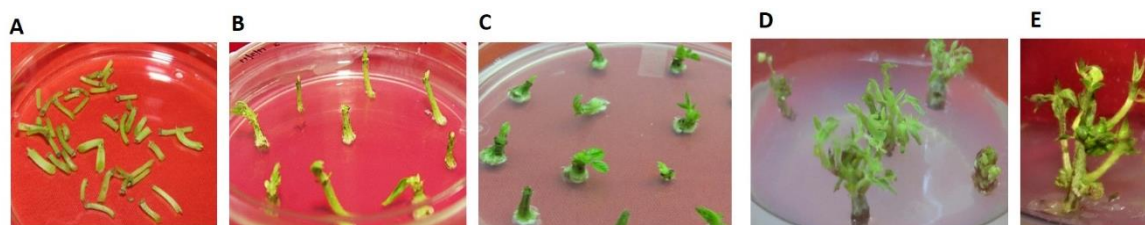


Figure 2. *Agrobacterium* -mediated transformation of greengram shoot tip explants using CRISPR construct A. Shoot tips excised from invitro germinated seeding B. Co-cultivated shoot tips explants with the CRISPR construct C. explants inoculated on kanamycin media for selection D. kanamycin resistant plants growing on regeneration media E. regenerated plants grown on elongation media for explant elongation

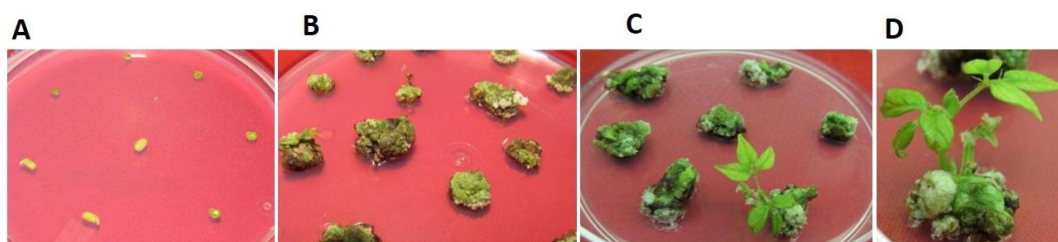


Figure 3. *Agrobacterium*-mediated transformation of greengram immature cotyledon explant using CRISPR construct A. immature cotyledons are grown with CRISPR construct B. the selection of embryogenic callus on kanamycin medium C. kanamycin resistant plants from calli grown on regeneration medium D. regenerated plants from calli were grown on elongation medium for plant elongation

The number of plantlets produced by a tissue culture independent *Agrobacterium tumefaciens*-mediated transformation by using three explants with transformation frequency with CN is 6.6 % with 8 plantlets, ST is 3.3 % with 4 plantlets and IMC is 4.1 % with 5 plantlets, therefore CN is a feasible method to generate transgenic plants in greengram with shoot and roots. The plantlets were transferred to glasshouse for further hardening (Figure 4 and Table 2).

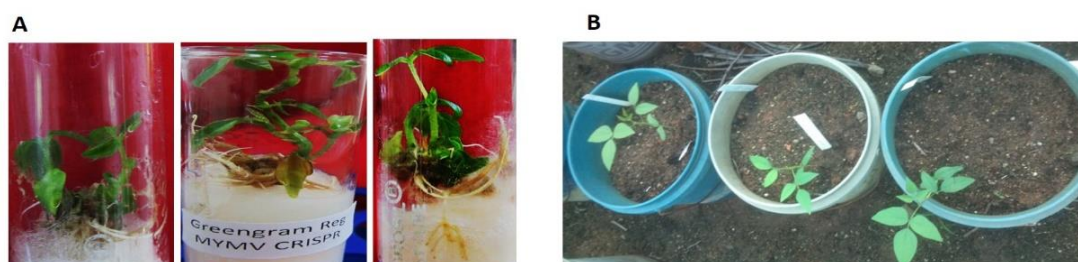


Figure 4. Different stages of shoot multiplication with rooting of three explants of green gram. A. Different explants which are regenerated, rooted after 2 weeks in liquid MSB5 + 1mg/L IBA medium B. Well established mature plants

Table 2. The greengram explants at different stages of transformation by using three various explants for regeneration and molecular analysis data of PCR

S.No	Greengram explants	infected in two batches (~60/per batch) No of explants	1st Round selection	2nd Round selection	No of plantlets revived in glass house	Percentage of explants regenerated on selection medium (T ₀)	PCR positive for npt II, Cas9
1	Cotyledonary node	120	53	32	8	6.60	5
2	Shoot tip	120	49	28	4	3.30	4
3	Immature cotyledons	120	42	15	5	4.10	5

Similar kind of results was also found by Yadav et al. (2010). In the present study we have observed that BAP enhances the multiple shoot induction with irrespective of explants and this frequency of regeneration was in accordance with the studies from (Yadav et al., 2010).

3.2 Molecular screening of regenerated plants by PCR

PCR analysis was performed on all of the regenerated and hardened plants to confirm the presence of *nptII* and *Cas9* genes using specific primers with amplicon size of 730 bp and 611 bp respectively. (Figure 5).

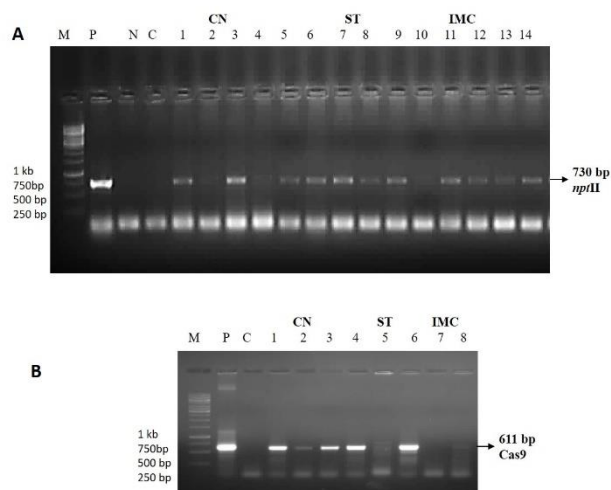


Figure 5. PCR Confirmation of putative transformed (T_0) plants: The gel picture showing the amplification of (A) *npt II* of 730 bp (1-14) and (B) *Cas9* of 611 bp (1-8) M; 1 Kb DNA marker, P; positive plasmid as template, N; negative control, C; Untransformed control: 1-14 (a); 1-8 (b) transformed greengram (T_0) plants. (CN-cotyledonary node, ST-shoot tip and IMC-immature cotyledon)

The frequency of transformation by PCR amplification was found as 6.6 %, 3.33 % and 4.1 % for CN, ST and IMC explants respectively (Table 2). The large number of escapes found during the selection process, which could be tolerant nature of *Vigna* spp. to the antibiotic kanamycin. Untransformed plants didn't show any amplification in the PCR. Among all three explants studied, CN explants ensured best suitable for raising the CRISPR-transgenic plants as it showed high transformation frequency. Although, IMC and CN explants were showed more or less equal transformation frequency. The method used for regeneration using immature cotyledon was indirect transformation *via* callus formation where the chances for somaclonal variations can take place (Sainger et al., 2015). Hence, the present study highlighted the cotyledonary node as the promising explant for generating CRISPR/Cas 9 based crop plants. The added advantage for using the CN as explant is its direct regeneration from meristematic tissue and no chance of somaclonal variations and required less time. In case of CN, the regeneration was direct, no somaclonal variations and the time required for establishing a plant in pot is less compared to other explants.

4. Conclusion

In this study, CN, ST and IMC explants were successfully used to generate effective CRISPR/Cas 9 based transgenic green gram plants and confirmed with PCR amplification. Among all the three types of explants tested cotyledonary node was remarkable in showing an efficient transformation of 6.6 % comparative to shoot tips and immature cotyledons. In IMC, embryonic calli production was lacking and in shoot tip, survival rate was decreased at selection stages. Thus in greengram CN explant regeneration on $MSB_5+0.5$ mg/L BAP +50 mg/L kanamycin can efficiently produce CRISPR-Cas9 transformed plants.

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Author's declaration and contribution

The authors certify that they have no conflicts of interest. MG: conceived project, framed experiments and approaches on conducting experiments, SA: suggestion and editing & review of the MS, AT: carried out all experiments and written MS and ND: review of the MS with suggestions for MS improvement.

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