

## Research Article

# Establishment of High Frequency Callus induction and Genetic Transformation in Neglected Leafy Vegetable *Amaranthus trisis*

Murugan SB<sup>1,2</sup> and Sathishkumar R<sup>1\*</sup><sup>1</sup>Department of Biotechnology, Bharathiar University, India<sup>2</sup>Department of Biotechnology, P.S.R. Engineering College, India

**\*Corresponding author:** Ramalingam Sathishkumar, Plant Genetic Engineering Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India

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**Abstract**

In the present study, an efficient *in vitro* callus induction and genetic transformation protocol was optimized for neglected leafy vegetable *Amaranthus trisis*. *In vitro* grown plant leaves were used as explants for callus initiation. Six different hormone combinations were tested for callus induction. Of the different hormone combination tested, Murashige and Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid and 6-benzylaminopurine (0.5mg/l) showed effective callus proliferation (87%). The frequency of callus induction ranged from 30.3% to 87%. Even though different hormone combinations were tried, the proliferated callus failed to differentiate into shoots. After 5<sup>th</sup> sub-culture, the callus was turned brown and dried. For genetic transformation, the plasmid pCAMBIA1301 harboring the *GUS* gene was transformed into the leaf explants by *Agrobacterium* mediated method. The presence of *GUS* gene in the putative transformed callus was confirmed by Polymerase Chain Reaction (PCR) and *GUS* histochemical staining. In PCR, an expected band of 1.2kb size was observed in all transformed calli confirming the presence of *GUS* gene. In histochemical staining, the transformed callus develops blue color in the presence of X-Gluc (5-Bromo-4-Chloro-3-Indolyl-Beta-D-Glucuronidase) reagent. Although shoot regeneration was unsuccessful, the present study might help to produce secondary metabolites from callus suspension cultures in *Amaranthus trisis*. This study can also prelude for metabolic engineering studies in this neglected vegetable to meet our future nutritional requirements.

**Keywords:** *Amaranthus trisis*; *Agrobacterium* transformation; Callus; Histochemical Staining; PCR

**Introduction**

Leafy vegetables are known to possess several ethno-medicinal properties, hence they are regarded as “Nature’s Anti-aging Wonders” [1,2]. The leafy vegetable *Amaranthus trisis* belongs to the family *Amaranthaceae*. The plants of this family are herbaceous, hard and fast growing plants. The leaves of this plant are edible and known to possess numerous medicinal properties [3]. In Ayurvedic system of medicine, it is stated that *Amaranthus* has potential medicinal properties and various species of this plant are used for curing several diseases. *A. tricolor* is used for quick healing of wounds and *A. spinosus* is used for treating diarrhea [4,5]. *A. trisis* is used as an astringent in dysentery and also used for treating cough and bronchitis [3]. In order to meet the daily sustainable food requirements this vegetables should be included in our diet.

The genetic engineering of plants has become the core research in plant molecular biology. The transformation efficiency determines the success in transgenic research. The genetic transformation of plants with the aim to introduce specific traits in plants has led to the development of crops with high nutritional value, resistance to biotic and abiotic stresses [6,7]. Totipotency of plant cell makes the genetic transformation and regeneration of plants easy. For the large scale transformation, it is necessary for the stable integration of foreign gene in genome of the organisms. Different transformation protocols

have been used for different species [8]. There are several methods available for the genetic transformation in plants *viz.*, *Agrobacterium* mediated, particle bombardment technology, microinjection, polyethylene glycol-mediated transfer and electroporation are the some of the methods used for the production of transgenic plants. Of these, *Agrobacterium* mediated and particle bombardment technology is the method of choice by most of the researchers for the genetic engineering experiments [9].

Establishment of *in vitro* system is necessary for both plant improvement and to conserve natural resources from extinction. As *in vitro* tissue culture was genotype dependent and also varies even between the cultivars, our aim was to optimize the protocol for high frequency callus induction and genetic transformation in *Amaranthus trisis* CO<sub>3</sub>, a valuable yet neglected leafy vegetable. As often vegetable crops are the ideal target for nutritional content improvement, this work could be a prelude for metabolic engineering approaches to meet our future nutritional requirements.

**Materials and Methods****Surface sterilization and seed germination**

The seeds of *Amaranthus trisis* variety CO<sub>3</sub> were procured from Seeds Certification Centre, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. The *Amaranthus* seeds were washed thrice

thoroughly with sterile distilled water, surface sterilized using 0.1% mercuric chloride and further washed with sterile distilled water to remove traces of mercuric chloride. The surface sterilized seeds were blot dried on sterile filter paper and aseptically inoculated in MS medium devoid of any hormones. The seeds inoculated were kept in dark for 3 days and then incubated in the light for 16±8 hours.

### Pre-culture of leaves and callus induction

*In vitro* grown plant leaves were used as explants in this study. The leaves were cut into small pieces and inoculated on pre-culture media. Six different hormone concentrations in different combinations were tried for effective callus initiation and proliferation. MS media supplemented with 2, 4-Dichlorophenoxyacetic Acid (2,4-D) and 6-Benzylaminopurine (BAP) (0.5 mg/L); Naphthaleneacetic Acid (NAA) and 6-Benzylaminopurine (BAP) (0.5 mg/L); 2,4-D and BAP (1mg/L), NAA and BAP (1mg/L), 2,4 -D and Kinetin (0.5 mg/L) and NAA and Kinetin (0.5 mg/L) were used. The leaves were pre-cultured on the above media for the callus induction.

### Agrobacterium mediated genetic transformation using pCAMBIA 1301

The pre-cultured leaves were used as explants for the DNA delivery studies in order to optimize the genetic transformation in *Amaranthus*. *Agrobacterium tumefaciens* strain EHA 105 harboring pCAMBIA 1301 was inoculated in Luria Bertani (LB) medium containing appropriate antibiotics and incubated overnight at room temperature with shaking. After reaching the OD of 0.6 at 600nm, the cells were harvested and pelleted at 5,000 rpm for 10 minutes and resuspended in liquid MS medium. The explants were infected for 10-15 minutes; blot dried on sterile filter paper for 20 minutes and were transferred to co-cultivation medium and incubated in dark condition for 3 days. After the co-cultivation in dark for three days, the explants were blot dried on sterile filter paper and inoculated on selection medium. Selection media was the same as the propagation media supplemented with 100mg/L Hygromycin + 300mg/L Carbenicillin (Duchefa, Germany).

### Screening of transformants

After two to three rounds of subculture in the selection medium, the genomic DNA from putative transformed callus was isolated [10]. In order to confirm the putative transformants, polymerase chain reaction was performed for *GUS* gene with gene specific primers. The *GUS* gene expression in transformed callus was analyzed by *GUS* histochemical staining. To the gently scrapped callus, X- Gluc (5-Bromo-4- Chloro-3- Indolyl- Beta-D- Glucuronidase) reagent was added and incubated in dark for 3-4 hours at 37°C. The transformed callus was observed for the development of blue color.

## Results

*Amaranthus* seeds were surface sterilized and inoculated on MS medium. Inoculated seeds were germinated after 7 days. The *in vitro* grown leaves of *A. tristis* were used as explants for callus formation. MS medium supplemented with different hormones in different combinations were tested for effective callus initiation. The initiation of callus was observed on the cut ends of the leaves after 10 days of the culture. Callus induction frequency was highly varied between hormonal combinations tested. Of the different hormone concentration tested, MS medium supplemented with 2, 4-D and



**Figure 1:** Callus formation in MS media containing 0.5 mg/l 2,4-D and 0.5 mg/l BAP.

**Table 1:** Callus responses of *Amaranthus tristis* Variety CO<sub>3</sub> in MS media with different hormonal combinations.

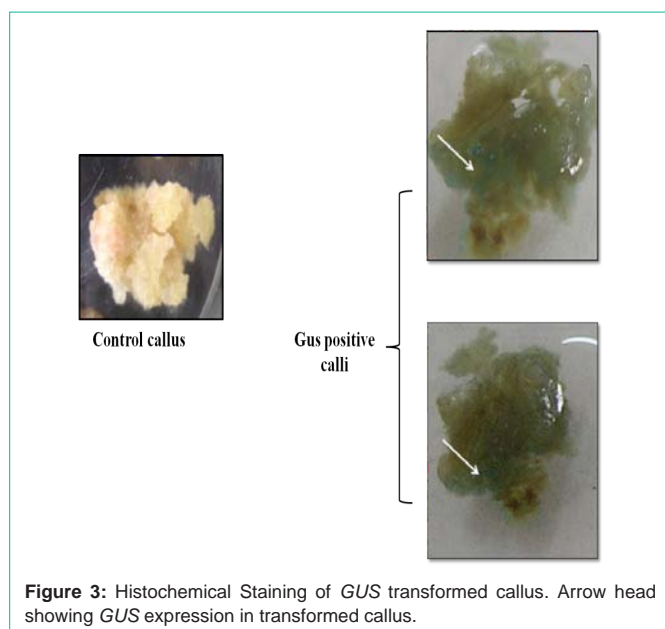
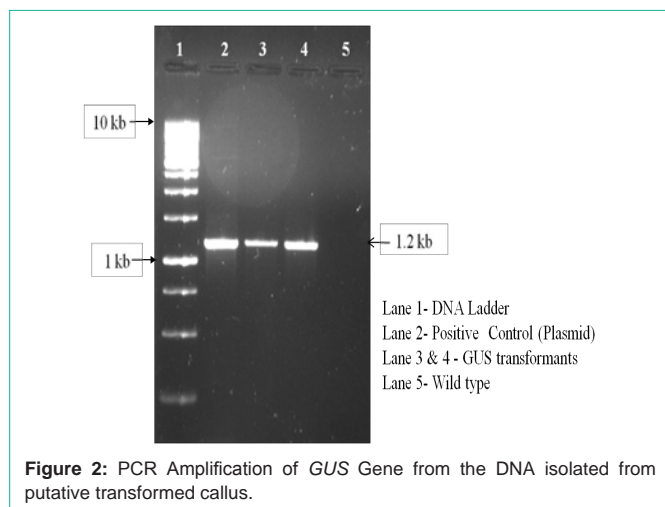
| Explant used             | Medium                     | Percentage of callus induction (%) |
|--------------------------|----------------------------|------------------------------------|
| <i>Amaranthus</i> leaves | MS+2,4-D+BAP (0.5mg/l)     | 87±0.56                            |
|                          | MS+NAA+BAP (0.5mg/l)       | 71.4±0.58                          |
|                          | MS+2,4-D+BAP (1mg/l)       | 68.75±0.58                         |
|                          | MS+NAA+BAP (1mg/l)         | 60.6±1.15                          |
|                          | MS+2,4-D+Kinetin (0.5mg/l) | 33.3±1.00                          |
|                          | MS+NAA+Kinetin (0.5mg/l)   | 30.3±0.52                          |

BAP (0.5mg/l) showed effective callus proliferation (87%) (Figure 1). The explants inoculated on MS medium with NAA and Kinetin (0.5mg/l) showed lowest callus induction percentage. The frequency of callus induction ranged from 30.3% to 87% (Table 1). Our results showed that the leaf explants of *A. tristis* cultured on MS medium supplemented either with 2, 4 D or NAA in combination with BAP produced more than 50% of callus. In all the tested combination, loose, white and rapid proliferation of callus was observed on callus induction medium after 3 weeks however no shoot differentiation and plant regeneration was observed. The necrosis was observed in the callus after 5<sup>th</sup> sub-culture and dried slowly with rooting.

In order to optimize the DNA delivery efficiency in *Amaranthus*, *Agrobacterium* mediated transformation was performed. The pre-cultured *Amaranthus* leaves were used for *Agrobacterium* transformation. For *Agrobacterium* mediated transformation, the co-cultivation period for 3 days has shown better results in *Amaranthus*. DNA isolated from putative transformed calli was confirmed by PCR by using *GUS* gene specific primers. The putative transformants were confirmed by the presence of specific band at 1.2kb which corresponds to *GUS* gene. The *GUS* gene expression was also confirmed by *GUS* histochemical staining (Figure 2 and 3).

## Discussion

*Amaranthus* is one of the important leafy vegetable that has high nutritional value which can be found throughout the tropics [11]. As it is underutilized vegetable, development of *in vitro* culture might helpful for its improvement and genetic manipulation. The callus induction is considered as the most important initial step for plant genetic transformation. In our study, 2, 4 D or NAA in combination of BAP produce white, loose callus. 2, 4 D is considered to be effective in callus induction. The effective callus formation and regeneration is considered as species specific [12]. Jofre-Garfias et al. [13] reported



that mature embryos of *Amaranthus hypochondriacus* cultured on MS medium supplemented with 10  $\mu$ M 2, 4-dichlorophenoxyacetic acid or 3, 6-dichloro-2-methoxybenzoic acid and 10% coconut liquid endosperm showed effective callus proliferation and plant regeneration. In our study, after 5<sup>th</sup> culture, callus turned brown and dried. This might be due to the accumulation of phenolics which inhibits the plant cellular growth [14,15].

The pre-cultured leaves were used for genetic transformation. Our study proved that co-cultivation period of 3 days resulted in high transformation efficiency; however co-cultivation period of above 3 days resulted in overgrowth of *Agrobacterium* and co-cultivation period of below 3 days result in low transformation efficiency. It has been reported that co-cultivation time varies between different species. Cervera et al. [16] reported that the co-cultivation period of 5 days showed maximum transformation efficiency in *citrange*. *Agrobacterium* mediated transformation through floral dip method in *Amaranthus* was reported earlier [17]. The major advantage of *Agrobacterium* mediated transformation was the integration of target

gene in the transcriptionally active regions of the chromosome and the integrated gene follows mendelian inheritance [18-20]. Initially this method was thought to be more suitable for dicotyledonous plants but this method has also been used to transfer the foreign gene in monocotyledonous plants like rice and banana [21,22]. Though there are advantages and disadvantages in all transformation methods, it is necessary to optimize the parameters based on the host system and the transformation method used. Once an effective transformation protocol can be optimized, the genetic manipulation can be made easy, more convenient and reproducible.

## Conclusion

In conclusion, we have optimized the hormonal combination for efficient callus induction and genetic transformation for neglected leafy vegetable *Amaranthus tristis* which would be very useful for the genetic improvement studies. Further studies are essential to find out the optimum media and hormonal concentration for the efficient regeneration system which can be prelude for metabolic engineering studies in this neglected vegetable to meet our future nutritional requirements.

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