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In planta transformation studies in *Gossypium hirsutum* (L.)

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Abstract
"*In-planta*" transformation method is a new method of transgenic development, where *Agrobacterium* is used to infect the plantlets devoid of *in vitro* regeneration. The preliminary findings of in planta transformation in *Gossypium hirsutum* (L.) suggested that the boll setting rate can be enhanced when liquid *Agrobacterium* culture with 5 percent sucrose was applied to the injured place in case of shoot tip, mature embryo and stigmatic surface in case of floral drip method. A single plant showed kanamycin resistance when injured embryo dipped in inoculums for 15 min was confirmed for the presence of *npt-II* gene specific band but no amplification was observed.

Keywords: *In planta* transformation, *Gossypium hirsutum*, transformation, shoot tip, floral dip, embryo infection

Introduction
In India, there are considerable economic losses in cotton production because of American bollworm (*Helicoverpa armigera*), pink bollworm (*Pectinophora gossypiella*), spotted bollworm (*Erias vitella*) and different sucking pests like jassids, aphids and whitefly. Sucking pest damage the crop in vegetative stage whereas different bollworms and whitefly nuisance the crop during reproductive phase of the crop [1].

The three cotton bollworms being internal feeders are difficult to control and thrive on fruiting parts of the crop, hence result in heavy economic losses. It is estimated that together the three bollworms can cause yield losses up to 80% in cotton. Resistance to almost all groups of insecticides has led to persistent insect control problems especially with *H. armigera* thus necessitating the need for viable alternative methods. Insect resistant transgenic cotton is perceived as one amongst the tools available to strengthen the integrated pest management programmes [2].

Incorporation of foreign DNA into plants has been achieved using the Ti plasmid of *Agrobacterium* tumefaciens or by direct gene transfer methods such as electroporation, PEG (polyethylene glycol) and microprojectile bombardment. Selectable markers are used to screen the foreign gene expression to confirm stable transformation [2].

The conventional transformation method typically includes preparation of transformation-competent plant cells or tissues, delivery into plant cells of foreign genes mainly by *Agrobacterium tumefaciens* or by the direct gene transfer method, selection of transformed cells that have stably incorporated foreign genes, and regeneration of transformed cells into transgenic plants. This transformation method has been widely used to produce the transgenic plants including many agriculturally important crops, whose tissue culture systems are well established. As this method involves tissue culture and plant regeneration step, this approach is painstaking and time consuming. It also requires skilled labour and relatively expensive laboratory facilities for its execution. Further, this method can result in undesired DNA modification and somaclonal variation during the processes of plant dedifferentiation and differentiation, which is mostly due to the stress imposed by the in vitro cell culture protocol [3].

One more major constraint in cotton improvement is of recalcitrance. Therefore, alternate methods that avoid/minimize tissue culture would be beneficial for the improvement of cotton. In this method, transgenic cotton plants have been produced by *in planta* transformation method. The elimination of tissue culture and regeneration greatly reduces hands-on-time, and success can be achieved by non-experts. Transformed plants can be obtained at sufficiently high rates so that the procedures can be used not only to introduce specific gene constructs into plants, but also as a random mutagenesis method for gene tagging [4].

Plant transformation method that excludes the use of tissue culture and plant regeneration was first described as *"in planta"* transformation almost 20 years ago by Xiuren and co-workers in 2006 [3]. Here we describe a novel non-tissue culture based approach for transformation of cotton (*Gossypium hirsutum* (L). which involved simple *in planta* inoculation of *Agrobacterium* solution onto cotton shoot tips and mature embryos which may give rise to stably transformed cotton plant.

Development of crop cultivar with insect resistance can form the backbone for integrated management of this pest. Stable introgression also offers strategies for over expressing or suppressing endogenous genes. The findings may guide future efforts to improve transformation of other plant species.

Material and Methods

PKV-Rajat is the hirsutum hybrid released by Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is moderately tolerant to bollworm and recommended for Vidarbha region used for the present investigation. Now days, as bollworms are acquiring resistance due to continuous use of synthetic pesticides, the cultivar shows remarkable pest attack thus reducing the yield. *Agrobacterium* is directed towards either the apical meristem or the meristems of axillary buds. The strategy essentially involves *in planta* inoculation of embryo axis of germinating seeds or ovary and allowing them to grow into seedlings *ex vitro*.

Shoot tip infection

In case of shoot tip infection, primary infection was done at seedling stage in 'pro' tray and secondary infection was done at branching stage to obtain any transformant branch.

Embryo infection

Bold and healthy seeds were sterilized with 0.01 per cent (W/V) mercuric chloride for 4 minutes, washed 3-4 times with sterile distilled water and soaked overnight. Next day, embryos were excised and precultured for 2 days on half strength Murashige and Skoog's medium in petri plates in culture room. Embryo was infected in three ways for *in planta* transformation as given below and tested for boll setting rate are-

1. Pricking of embryo with needle dipped in bacterial culture
2. Dipping injured embryo in bacterial culture for 15 min
3. Dipping injured embryo in bacterial culture for overnight

The infected embryos were germinated in soil rite: cocopit (1:1) under controlled temperature and humidity.

Floral dip method

For the floral dip method the plants were grown in pot in transgenic green house. When the flowering starts, the flowers were treated with liquid *Agrobacterium* culture containing 5 percent sucrose and acetosyringone @ 100 µM. The treated flowers were bagged using butter paper bag in order to avoid cross pollination chances.

Effect of state of agrobacterial culture on the boll set

Solid and liquid culture of *Agrobacterium* was used for pollination for *in planta* transformation at evening of the same day or at next day morning. The observations were recorded for boll set after pollination. For solid culture, the colonies were scratched from Yeast Extract Mannitol Agar plates whereas in case of liquid culture the pellet from Yeast Extract Mannitol Agar broth was obtained after centrifugation and dissolved in ½ MS broth containing acetosyringone @ 100 µM to increase transformation rate.

Effect of sucrose in agrobacterial culture while infection on the boll set

Direct application of agrobacterial culture to stigmatic surfaces reported very poor boll setting. Therefore, forty eight hrs grown liquid bacterial culture along with sucrose @ 5% was used to infect stigma of the flower at evening of the same day or at next day morning. The observations were recorded for boll set after pollination.

Result and Discussion

***In planta Agrobacterium* mediated transformation**

A preliminary study was carried out for *in planta* transformation in order to know boll setting rate with different methods available. These are advantageous over in vitro transformation methods as they are bereft of inherent problems associated with tissue cultural procedures. With a view to harness these benefits, *in planta* cotton transformation was attempted in this study.

Prior to go for *in planta* transformation, some parameters such as effect of state of *Agrobacterium* culture (solid/liquid), effect of duration of infection and effect of sucrose in agro-inoculum on boll setting was optimized.

Effect of state of agrobacterial culture on the boll set

From the table 1 it is clear that pollination made on next day morning with liquid culture and yielded two bolls out of 100 pollinations. So, for further study the same treatment was attempted.

Table 1: Effect of state of *Agrobacterium* culture on boll set

S. No	State of *Agrobacterium* culture used	Time of emasculation and Application of *Agrobacterium*	Time of pollination	No. of bolls Set
1	Solid culture	Evening	On the same evening	Nil
2	Liquid culture	Evening	On the same evening	Nil
3	Solid culture	Evening	Next day morning	Nil
4	Liquid culture	Evening	Next day morning	02

Boll set was significantly higher (28.5%) when the plants were infected with liquid *Agrobacterium* culture when compared to the boll set resulting from the use of solid *Agrobacterium* culture. This difference may be due to the fact that the bacterial load is more in solid culture because of higher inoculum density resulting in decreased cell viability. These observations appear to give credence to the hypothesis that each plant cell binds to a finite number of bacteria. Moreover, from the broth bacteria can move with the pollen tube path more easily than from agar medium and reach the embryo sac to effect fertilization. This indicates that the precise guidance of the pollen tube to the embryo sac is critical to the successful sexual reproduction in flowering plants [5].

Effect of application of sucrose to stigmatic surface on the boll set

Various methods attempted to achieve successful boll setting with direct application of agrobacterial culture to stigmatic surfaces resulted in poor boll setting (5 percent). Most of the pollinations were abortive and they were shed 3-4 days after fertilization, so the stigmatic surface was treated with 5 percent sucrose solution, which resulted in significantly higher boll set percent (11 percent). The results are presented in table no. 2.

Table 2: Effect of application of sucrose to stigmatic surface on the boll set in cotton

S. No	Treatment	No. of pollinations carried out	Percent boll set
1	Without Sucrose	100	05
2	With 5 percent Sucrose	100	11

Tetsuya and co-workers in 1998 have reported that sucrose concentration of 5 per cent appeared to be optimum for pollen tube growth also achieved improvement in the growth of pollen tubes by increasing the amount of boric acid from 0.5 to 10 mg/l [6]. It was reported that sucrose level was more crucial for the pollen penetration. Further there was marginal increase in rate of boll set by addition of boron. Encouraged by the results of sucrose and boric acid inclusion in the treatment, combined effect of both were tried in subsequent experiments and improvement in the boll set was obtained [7].

Different methods for *in planta* transformation

Shoot tip infection

When the primary infection was made to seedling grown in 'po' trays as shown in fig. 1(A), not single seedling survived after 8-10 days. In case of secondary infection shown in fig. 2(B), the injury was made to the seedling grown in transgenic house at nodal portion/branching point also did not yielded any branching further.

Reddy in 2007 when tried *cry*1Ac gene transfer, in T0 generation of chickpea the lowest incubation period (10 min) resulted in higher kanamycin resistant plants (4 out of 32), followed by 20 min incubation period (3 out of 30). The least number of kanamycin resistant plants were obtained in incubation period of 30 min (1 out of 25). In T1 generation plants obtained from 30 min incubation period did not show resistance to kanamycin. One plant in each incubation periods of 10 min and 20 min showed resistance to kanamycin [8].

Fig 1: *In planta* transformation using shoot tip injury method, Primary Infection (A) & Secondary Infection (B)

Embryo infection

When embryo infected with needle just dipped in inoculums, the regeneration was higher but none of the plants showed kanamycin resistance. As injured embryos were infected with *Agrobacterium* culture for 15 min, one kanamycin resistant plant was obtained and overnight colonization of embryo with inoculums also did not yield any kanamycin resistant plant (Table 3).

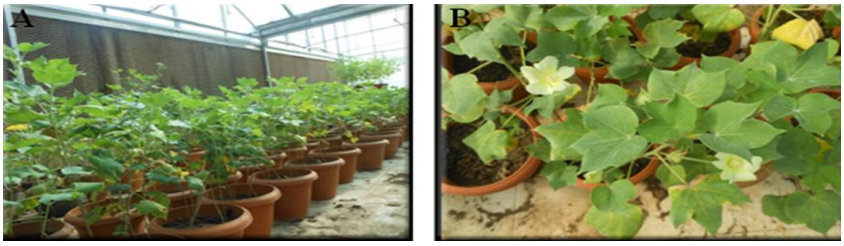
Table 3: Effect of pricking embryos on *in planta* transfer of *cry*1F gene

Treatment	Number of Embryos treated	Number of Seedlings established	Reaction of T0 generation plants to kanamycin	
			Resistant	Susceptible
Pricking with needle dipped in inoculums	50	27	0	27
Dipping injured embryo in inoculums	50	15	1	14
Dipping in inoculation for overnight shaker	50	2	0	2

Embryo axes with one of the cotyledon wounded by pricking with a needle were subjected to *Agrobacterium* infection to produce transgenics in Arabidospsis [9], soybean [10], in peanut, safflower and sunflower [11] and rice and wheat [12] have earlier demonstrated *in planta* methods of similar nature. Rohini and Rao (2000) followed *in planta* method by pricking embryos and dipped in inoculum for 10 min with intermittent shaking in peanut. Hundred embryos treated in this way grew (T0 generation) to maturity and advanced to next generation. In T1 generation, three transformation events were confirmed out of 89 plants [11]. Embryos were pricked at apical meristem and cotyledonary node with needle dipped in inoculum. Hundred embryos treated in this way grew (T0 generation) to maturity and advanced to next generation. In T1 generation two transformation events were confirmed out of 42 plants in mulberry [13]. In Hibiscus sabdarifa, pricked embryos were dipped in inoculum and kept on shaker overnight yielded few viable seedlings which were kanamycin susceptible [14]. Supartana *et al* in 2006 developed *in planta* transformation method for wheat by soaking seeds in water at 22 ^{0}C for 1 day and then piercing the region of embryonic apical meristems with needle dipped in *Agrobacterium* inoculum. The transformation efficiency of T1 plants up to 33 percent was estimated by PCR analysis, 75 percent by southern hybridization and 40 percent by plasmid rescue [12].

Floral dip method

Hundred flowers were treated with liquid *Agrobacterium* culture containing 5 percent sucrose and acetosyringone @ 100 uM. The boll setting was observed but shredded during later stages of development as shown in fig 2.



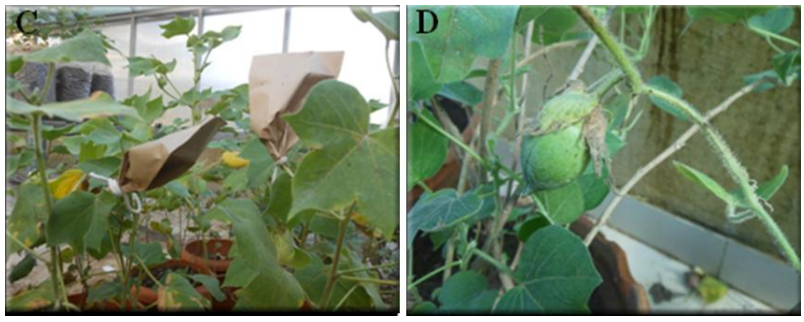


Fig 2: *In planta* transformation using floral dip method, plants grown in transgenic house (A), Plants at flowering stage (B), flowers bagged after floral dip (C), Boll setting after flower dip (D)

Clough and Bent in 1998 developed transformation method by simply dipping of floral tissues into a solution containing *Agrobacterium* tumefaciens, 5% sucrose and 500 µl/lit silwet L-77. Plants inoculated when numerous immature floral buds and few siliques were present produced transformed progeny at the higher rate. Repeated application of *Agrobacterium* improved transformation rates and overall yield of transformants approximately twofold. Covering plants for 1 day to retain humidity after inoculation also raised transformation rates twofold [4]. Two rapid and simple *in planta* transformation methods were developed for the model legume Medicago trunculata. The first approach is based on a method developed for transformation of Arabidopsis thaliana and involves infiltration of flowering plants with *Agrobacterium*. The second method involves infiltration of young seedlings with *Agrobacterium*. In both cases the transformation frequency ranges from 4.7 to 76 percent for flower infiltration method, and from 2.9 to 27.6 percent for the seedling infiltration method [15]. Female gametes were transformed by simply dipping developing Arabidopsis inflorescences for a few seconds into a 5% sucrose solution containing 0.01–0.05% (v/v), silwet L-77 and re-suspended *Agrobacterium* cells, carrying the genes to be transferred. Treated plants were allowed to set seed which are then plated on a selective medium to screen for transformants. A transformation frequency of at least 1% can be routinely obtained and a minimum of several hundred independent transgenic lines generated from just two pots of infiltrated plants (20–30 plants per pot) within 2–3 months [16]. The transgenic cotton plants were developed by pistil dip inoculation in a solution containing *Agrobacterium* carrying a gene for resistance to the herbicide basta (bar), 10% (w/v) sucrose, 0.05% (v/v), silwet L-77 and 40 mg acetosyringone per litre. Pistil drip during 17:00–19:00 on the first day of flowering resulted in 0.07–0.17% basta-resistant plants/number of viable seeds generated, and stigma excision prior to pistil drip during this time period gave rise to a transformation efficiency of 0.46–0.93%, in contrast with 0.04–0.06% generated from pistil drip during 9:00–11:00 on the second day of flowering. PCR and Southern blot analysis confirmed the integration of the bar gene into the cotton genome, and a T1 and T2 generation herbicide resistance test consistently revealed expression and stable heritability of the bar gene in the two generations [17].

Confirmation of putative transformants

A single plant showed kanamycin resistance when injured embryo is dipped in inoculums for 15 minute and was confirmed for the presence of *npt-II* gene specific band. Though it showed kanamycin resistance, no amplification was seen when resolved on 1 percent agarose gel.

Conclusion

Furthermore different factors *in planta* transformation need to be investigated in cotton such as duration of co-cultivation period, Triton-X 100 concentrations, effects of vernalization and dehydration of seeds or embryos before co-cultivation period, effect of wounded leaf extracts and acetosyringone in inoculum, vacuum infiltration of well-established seedlings and flowers which may increase transformation efficiency. So that, more number of genes can be used for transgenic development in insect management programme.

References

1. Sundaramurthy VT, Jayaswal AP, Bhamburkar MW, Banerjee SK. Insect pests and their management in cotton. Basu AK, Mannikar ND, Narayanan SS (eds) Cotton scenario in India, A souvenir, ICAR, Pusa, New Delhi, India. 1990, 88-95.
2. Kranthi KR, Sandhya Kranthi, Dongre AB. Cotton biotechnology. CICR technical bulletin No. 10, Central Institute for Cotton Research, Nagpur, 2000.
3. Xiuren Zhang, Rossana Henriques, Shih-Shun Lin, Qi-Wen Niu, Nam-Hai Chua. *Agrobacterium*-mediated transformation of Arabidopsis thaliana using the floral dip method. Nature Protocol. 2006; 1(2):1-6.
4. Clough SJ, Bent AF. *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. The Plant J. 1998; 6(6):735-743.
5. Gutlitz RGH, Lamb PW, Matthsse AG. Involvement of carrot cell surface proteins in attachment of *Agrobacterium tumefaciens*. Pl. Physiol. 1987; 83:564.
6. Tetsuya H, Kuroiwa H, Shigeyuki K, Kuroiwa T. Guidance in vitro of the pollen tube to the neaked embryo sac of *Torenia fournieri*. Pl. Cell. 1998; 10:2019-2032.
7. Reddy N, Zhu Shuijin, Rong JY, Hong TX. Pollen tube pathway mediated genetic transformation of cotton. Proceedings of International Symposium on Strategies for Sustainable Cotton Production – A Global Vision". 23-25 November 2004, Univ. Agril. Sci., Dharwad, Karnataka, 2004.
8. Raddy YS. *In planta* transformation studies in chick pea (*Cicer arietinum* L.) M.Sc. thesis, Univ. Agric. Sci., Dharwad, 2007.
9. Feldmann KA, Marks MD. *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. Mol. Gen. Genet. 1987; 208:1-9.
10. Chee PP, Fober AK, Slightom LJ. Transformation of soybean (*Glycine max* L. (Merrill)) by infecting germinating seeds with *Agrobacterium tumefaciens*. Pl. Physiol. 1989; 91:1212-1218.

11. Rohini VK, Rao S. Transformation of peanut (*Arachis hypogaea* L.): a non-tissue culture based approach for generating transgenic plants. Pl. Sci. 2000; 150:41-49.
12. Supartana P, Shimizu T, Nogawa M, Shioiri H, Nakijima T, Haramoto N *et al*. Development of simple and efficient *in Planta* transformation method for wheat (*Triticum aestivum* L.) using *Agrobacterium tumefaciens*. J Biosci. Bioengg. 2006; 100(4):162-170.
13. Ping LX, Nogawa M, Nozue M, Makita M, Takeda M, Bao L *et al*. *In planta* transformation of mulberry trees (*Morus alba* L.) by Agrobactetium *tumefaciens*, J. Insect Biotech. Sericol. 2003; 72:177-184.
14. Yaye KG, Djibril S, Mansor N. Direct genetic transformation of *Hibiscus sabdariffa* L. African J Biotech. 2004; 3(4):226-228.
15. Trieu AT, Burleigh SH, Kardailsky IV, Maldonado-Mendoza IE, Versaw WK, Blaylock LA *et al*. Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. The Plant J. 2000; 22:531-541.
16. Zhang X, Henriques R, Lin S, Niu Q, Chua N. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nature Prot. 2006; 1(2):1-6.
17. TianZi C, ShenJie W, Zhao J, Wang Zhen G, Tian Zhen Z. Pistil drip following pollination: A simple *in planta* Agrobacterium-mediated transformation in cotton. Biotechnol. Lett. 2010; 32:547-555.