DISCUSSION
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*Sesamum indicum* L. is one of the most important oil crop. Seeds contain powerful antioxidant called Lignans (Sesamins), Saseolin and phytosterol which impart a high degree of resistance against oxidative rancidity among the other oil crops. *In vitro* callus induction, somatic embryogenesis, *in vitro* regeneration and genetic transformation through *Agrobacterium tumefaciens* has been worked out in sesame. But the ability of isolated plant cells or tissues to grow and divide in culture and regenerate into whole plant varies for different genotypes and it is foremost requirement for developing protocols for the above said aims for the production of transgenic plant resistant to biotic and abiotic stress. Though callus induction and organogenesis has been reported in many other varieties of *Sesamum* (Kwon *et al*., 1993; Taskin *et al*., 1997; Kariyallappa *et al*., 2003; Baskaran and Jayabal, 2006; Were *et al*., 2006; Shashidhara *et al*., 2011), there are no reports on this high yielding but disease prone varieties, hence for the genetic improvement of this variety the first and foremost requirement is to develop protocols for tissue culture and to standardize optimum requirement for *Agrobacterium tumefaciens* mediated transformation.

**Nature of the explant**

Callusing response of different explants varies according to the nature of the donor organ. It has been reported that different explants widely vary in their response callogenesis and organogenesis and this may be due to differences in spatial and temporal distribution of cells and their physiological, biochemical and developmental stages, further genetic makeup of plant is a major factor on which *in vitro* culture establishment
and morphogenetic behavior is dependent (Vasil and Vasil, 1986). Murashige (1974) recognized several factors that could be considered in the explant selection, these includes

1. Organ which is to serve as tissue source

2. Physiological and genetic age of the organ

3. Season in which explant is to be obtained

4. Size of the explant

5. The overall quality of the plant form which explants are to be obtained

**Influence of explants on callus induction**

In the present investigation, callus induction from two explants viz., cotyledon and hypocotyl using auxins alone or in combination with cytokinin is standardized. It is evident that, hypocotyl explant proved to be excellent choice, in terms of time required for callus induction frequency and growth of callus followed by cotyledon explant. There are several reports in *Sesamum* for callus induction (George et al., 1987; Lee et al., 1988; Rajender et al., 1997b; Saravanan and Nadarajan, 2005; Bhaskaran and Jayabal, 2006; Shashidara et al., 2011) and in other oil seeds viz., sunflower (Paterson and Everett, 1985; Piubello and Caso, 1986 and Liu et al., 1991), ground nut (Venkatachalam et al., 1997), soyabean (Hammett and Davey, 1988; Horn et al., 1992 and Horn and Widholm, 1994) and in mustard (Gupta et al., 1990) where hypocotyl is shown as better explant than cotyledon.
Influence of explants on multiple shoot induction

A wide varieties of explants have been used in *Sesamum* for induction of multiple shoots viz., deembryonated cotyledons (Seo et al., 2007 and Lokesha et al., 2012), cotyledon and hypocotyls (Taskin and Turgut, 1997; Gangopadhyaya et al., 1998; Kim 2001; Kariyallappa, 2003; Saravanan and Nadarajan 2005; Baskaran and Jayabalans, 2006; Shashidhara et al., 2008; Were et al., 2006; Bangaramma, 2009; Yadav et al., 2010; Chattopadhyaya et al., 2010). However in the present investigations hypocotyl, deembryonated cotyledons didn’t respond to organogenesis only the deembryonated seeds responded for organogenesis with high frequency (87.78± 2.6). These contradictory results may be due to the different varieties used by these workers and in the present investigations. Genotype depended response to organogenesis is reported in *Sesamum* by (Lee et al., 1988; Saravanan and Nadarajan, 2005) and in other oil seed crops like *Brassica* species (Moghaieb et al., 2006) Groundnut (Ozudogru et al., 2013), *Arachis paraguariensis* (Olubunmi et al., 2012), Sunflower (Tican et al., 2007, Rao and Ramgopal, 2010).

Influence of explants on somatic embryogenesis

From the survey of literature it is found that there are no reports of direct somatic embryogenesis from explants in sesame, however there are only two reports where callus mediated somatic embryogenesis is observed. Mary and Jayabalans, (1997) reported somatic embryogenesis from hypocotyl derived callus in sesame var. TMV-6, and that of Xu et al., (1997) who reported somatic embryogenesis from callus derived from different explants like cotyledons, root and sub apical hypocotyl segments in var. Nigrum.
**Influence of auxins on callus induction**

In the present studies hypocotyl explants responded well producing callus on medium supplemented with 1.0-5.0mg/l NAA. Callus induction in response to NAA is reported in sesame (Kwon et al., 1993; Lokesh et al., 2007; Baskarn and Jayabalan, 2006 and Shashidhara et al., 2011). NAA is considered to be a potent auxin for the induction of callus in several oil seed producing plant species (Rao and Ramgopal, 2010, Pandurang et al., 2012 Iqbal et al., 2011). In the present studies IAA failed to induce sufficient amount of callus and in 2, 4-D supplemented medium only somatic embryogenesis was observed. Bhaskaran and Jayabalan, (2006) also reported that 2, 4-D failed to induce sufficient amount of callus in sesame. However Saravanan and Nadarajan, (2005) reported callus induction in 4 varieties (CO 1, VRI 1, TMV 3 and AHT 123) of *Sesamum* on medium supplemented with 2, 4-D+ 100ml/l coconut milk. These contradictory results may be due to the different varieties used by these workers and in the present investigations where var. E-8 of sesame is used.

**Interaction of cytokinins and NAA on induction and growth of callus**

Cytokinins like BAP and Kn when added in small amounts to an auxin containing medium are reported to enhance the frequency of callus induction and enhanced growth of callus (Iqbal et al., 2011; Pandurang et al., 2012).

In the present investigations also it was notice that cytokinins like BAP and Kn. when supplemented to medium containing 2.0mg/l NAA enhanced the growth of callus in terms of fresh and dry weight as reported by Kwon et al., (1993), Lokesha et al.,
(2007) and Shashidhara et al., (2011) in this species. There are few reports where enhanced growth of callus is reported when BAP was supplemented to medium containing 2, 4-D in this species (Lee et al., 1985; George et al., 1987 and Rajender et al., 1997b), however in the present investigations on 2, 4-D+ BAP supplemented medium we observed formation of direct somatic embryos without callus formation, this contradicting result may be due to different varieties used. Similar trend of genotypic variation is noticed in several other oil seed producing plant species (Banerjee, 2013; Totik et al., 2014).

**Effect of BAP on induction of multiple shoots**

BAP is reported to induce multiple shoots in several plant species (Banerjee, 2013; Pandurang et al., 2012; Joel et al., 2013). In the present investigations BAP 1.0-6.0mg/l was able to induce multiple shoots from deembryonated seeds in sesame. There was gradual increase in the frequency and number of multiple shoots with an increase in the concentration of BAP maximum number being on medium supplemented with 5.0mg/l BAP and at 6.0mg/l the number of multiple shoots decreased. BAP mediated multiple shoot induction is reported in Sesamum by Chattopadhyaya et al., (2010), Seo et al., (2007), Gangopadhyay et al.,(1998), Abdellatef et al.,(2010), Yadav et al., (2010). TDZ is also reported to induce multiple shoots in this species (Lokesha et al., 2012).

**Effect of BAP and IAA on induction of multiple shoots**

IAA is reported to enhance multiple shoot formation in several plant species (Sujatha et al., 2012; Iqbal et al., 2011). In the present investigations also it was noticed
that supplementing IAA 0.5 to 1.0mg/l to BAP containing medium increased the frequency and number of multiple shoots maximum being at 1.0mg/l further increase in the concentration of IAA reduction in the number of multiple shoots. IAA is reported to increase the number of multiple shoots in sesame (Were et al., 2006; Seo et al., 2007 and Lokesha et al., 2012).

**Effect of supplementing ABA on induction of multiple shoot**

ABA is reported to enhance the multiple shoots formation in several plant species (Hoang and Raldugina, 2012; Rahman et al., 2010 Peterson and Smith, 1991). In the present investigations also it was noticed that supplementing ABA 0.5 to 1.0mg/l to BAP and IAA containing medium increased the frequency and number of multiple shoots maximum being at 1.0mg/l further increase in the concentration of ABA reduction in the number of multiple shoots. ABA is reported to increase the number of multiple shoots in sesame (Seo et al., 2007).

**Effect of supplementing silver nitrate (AgNO₃) on induction of multiple shoots:**

A number of researchers have reported that addition of silver nitrate to the culture medium promotes in vitro regeneration, possibly due to the compound’s anti-ethylene activity (Orlikowska, 1997) AgNO₃ has been known to inhibit ethylene action (Beyer, 1976a) . In recent years, AgNO₃ has been employed in tissue culture studies for inhibiting ethylene action because of its water solubility and lack of phytotoxicity at effective concentrations (Beyer, 1976a). In the present investigations it was noticed that supplementing AgNO₃ 1.0 to 5.0mg/l increased the frequency and number of multiple
shoots maximum being at 5.0mg/l further increase in the concentration of AgNO₃ gradual reduction in the number of multiple shoots. AgNO₃ is reported to increase the number of multiple shoots in sesame (Seo et al., 2007; Abdellatef et al., 2010).

**Elongation of *in vitro* raised shoots**

In the present investigation GA₃ 0.5-1.0mg/l increased the elongation of shoots further increase in the concentration elongation was declined. Similar reports were available on the elongation in sesame by Chattopadhyaya et al., 2010. In few other reports of sesame elongation achieved on rooting media containing NAA (Karimi et al., 2011) and low concentration of BAP has been used in combination with NAA (Gangopadhyaya et al., 1998). This contradicting report may be due to different varieties used by other workers.

**Rhizogenesis**

In the present investigation IBA used for the rooting of *in vitro* raised shoots and few other authors also reported the same in other oil seed crops (Elavazhagan, 2009; Fabijan et al., 1981; Shuster et al., 2011).

In the present investigation, *in vitro* rooting was observed on media containing IBA. However rhizogenesis on hormone free medium is reported in this species by Gangopadhyaya et al., 1998 and Chattopadhyaya et al., 2010. This contradicting report may be due to different varieties used by other workers. Saravanan and Nadarajan (2005) investigated rooting on medium supplemented with IAA + IBA. Abdellatef et al., (2010)
reported rooting on NAA and further added that enhancement of rooting was noted when, the rooting medium was further supplemented with AgNO$_3$ and Cobalt chloride.

**Somatic embryogenesis and conversion into plantlet**

Somatic embryos can be distinguished from adventitious shoots, because they are bipolar having both a shoot and root pole and they don’t have vascular connection with the underlying parental tissue and regeneration through somatic embryogenesis is preferred over organogenesis because of single cell origin of the somatic embryos (Merkel *et al.*, 1995).

In the present study direct somatic embryogenesis is noticed in cotyledon and hypocotyl explants on medium supplemented with 2, 4-D. Cotyledon responded better than hypocotyl explant. Addition of BAP to 2, 4-D enhanced the frequency and number of somatic embryos per explant. Reports are not available on direct somatic embryogenesis from explant without intervening callus phase in this plant. However indirect somatic embryogenesis from callus derived from cotyledon, root and subapical hypocotyl segments on MS medium supplemented with 2, 4-D is reported by Xu *et al.*, 1997 and hypocotyl derived callus by Mary and Jayabalan, 1997. Direct somatic embryogenesis was reported in few other plants using 2, 4- D (Kuo *et al.*, 2005; Girijashankar *et al.*, 2007).

Silver nitrate, a potent inhibitor of ethylene action Beyer, (1976a) was shown to improve somatic embryogenesis in tetraploid potato (Evans and Vatty, 1994) and Barley (Tiainen, 1992).
Conversion of somatic embryos

In the present study conversion of somatic embryos into complete plantlet was achieved on BAP, ABA and AgNO₃. Similar reports are available on the conversion of somatic embryos into complete plantlet in other species using same hormonal combination (Nasab et al., 2012, Rahman et al., 2010).

Genetic transformation

In the present study Agrobacterium tumefaciens mediated genetic transformation was carried out in sesame using AP37 gene meant for Salinity and Drought tolerance.

*Agrobacterium tumefaciens* mediated genetic transformation

Transformation of plants by *Agrobacterium* mediated DNA transfer in the most commonly used phenomenon in accomplishing plant gene transfer. Protocol have been developed for efficient *Agrobacterium tumefaciens* mediated transformation in both dicotyledonous and monocotyledonous plants, including a large number of crop species. The first transgenic plant expressing engineered foreign genes were tobacco plants produced by the use of *A. tumefaciens* vectors (Horsch et al., 1984; Deblock et al., 1984). Since then many plant species have been genetically engineered using *A. tumefaciens*.

In order to develop a good transformation protocol in sesame with a higher efficiency of response, various factors influence the transformation efficiency such as *Agrobacterium* cell population, type of *Agrobacterium* infections and hygromycin were studied.
The yield potential of sesame is very low when compared with major oil seed crops due to early senescence and extreme susceptibility to biotic and abiotic stress factors including photo sensitivity (Rajender et al., 2002). Wild species of sesame possess genes for resistance to biotic and abiotic stresses (Brar and Ahuja, 1971 and Kolte, 1985). However, introgression of useful genes from wild species into cultivars via conventional breeding has not been successful due to post fertilization barriers. The only option left for crop improvement of sesame is to transfer genes from other sources through genetic transformation techniques.

The main obstacle to genetic transformation is the recalcitrant nature of sesame to in vitro regeneration due to over production of secondary metabolites (Baskaran and Jayabalan 2006).

Since a reliable protocol is developed for tissue culture in sesame, therefore in the present study Agrobacterium tumefaciens mediated genetic transformation was carried out in sesame using AP37 gene meant for salinity and drought tolerance.

Plant genetic transformation has become an important tool for functional genomics and as an adjunct to conventional breeding programmes gene transfer by Agrobacterium is the established method of choice for the genetic transformation of most plant species. Agrobaecterium tumefaciens mediated transformation in both dicotyledonous and monocotyledonous plants, including a large number of crop species. The first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of A. tumefaciens vectors (Horsch, et al., 1984; De et al., 1984), since then many plant species have been genetically engineered using A. tumefaciens.
This method of transformation is perceived to have several advantages over other forms of transformation (such as biolistics), including the ability to transfer large segments of DNA with minimal rearrangement and with fewer copies of inserted genes at higher efficiencies with lower cost (Das et al., 2006). In addition, *Agrobacterium* transformation may facilitate the removal of plant selectable marker genes by segregation (Muller et al., 2002). *A. tumefaciens* mediated transformation in plant species relies on the availability of an efficient *in vitro* regeneration system amenable to genetic transformation, for developing transgenic plants in all crops.

Efficiency of *Agrobacterium*-mediated transformation and delivery of T-DNA into plant cells is influenced by several physico-chemical and physiological conditions such as type of explant, bacterial density, co-cultivation period, pre-culture, sensitivity to antibiotics, concentration of acetosyringone. Present study focuses on the optimization of mentioned conditions in sesame transformation.

In the present investigation *Agrobacterium* mediated transformation protocols for deembryonated seeds was developed. *Agrobacterium tumefaciens* strain LBA 4404 strain is used which carries a super binary vector with AP37 gene, GUS gene and hpt-II gene. LBA 4404 strains have been routinely used in many laboratories all over the world for plant transformation in several crop species (Muller et al., 2001; An et al., 2014)

**Type of explants**

Attachment of the bacterium to the host plant cell wall is an initial step in the process of infection and it can be affected by plant or tissue age, cell type, cell cycle stage
and other physiological parameters (Graves et al., 1998). In our experiments the
deembryonated seed explant was chosen in the transformation studies.

**Optimization of lethal dose of Antibiotics**

Antibiotics play a very vital role in transformation where the use antibiotics
depends on the makeup of *Agrobacterium* strain, accordingly in present transformation
study two antibiotics were used namely hygromycin as a selection marker, cefotaxime is
to minimize the over growth of bacteria. The successful elimination of *Agrobacterium*
from the regeneration media in transformation protocols is important for the successful
recovery of transgenic tissues. Antibiotics such as cefotaxime, carbenecillin and timentin
have been used regularly in *Agrobacterium*- mediated transformation of crops following
co-culture to suppress or eliminate *Agrobacterium* (Cheng et al., 1996; Bottinger et al.,
2001; Sunilkumar and Rathore, 2001). However Taskin et al., 1999 and Yadav et al.,
2010 used Kanamycin as selection marker and Rifampisin to control the bacterial growth.

**Optimization of lethal dose of cefotaxime**

The most commonly used antibiotic to control the *Agrobacterium* growth is
cefotaxime. Cefotaxime has a broad spectrum activity against both gram positive and
gram negative bacteria and functions by blocking the cell wall mucopeptide biosynthesis,
by inhibiting the cross-linking of peptidoglycan and by binding and inactivating of
transpeptidases. Before transformation different concentration (0, 50, 100, 150, 200 and
250mg/l) of cefotaxime were tested by disc diffusion assay to inhibit the growth of
*Agrobacterium*. From the results it was observed that 250mg/l of cefotaxime showed
higher area of bacterial inhibition. The results demonstrated that 250mg/l cefotaxime was optimum concentration for controlling the growth of Agrobacterium. Several reports are available on the concentration of cefotaxime used to control the over growth of Agrobacterium. Tripathi et al., (2005, 2008) used 300 and 500mg/l cefotaxime, Maziah et al., (2007) reported use of 600mg/l cefotaxime for controlling the over growth of bacteria.

**Optimization of lethal dose of hygromycin**

Hygromycin is a member of the aminoglycosides antibiotics, which have been extensively studied for its effect on tissues. Hygromycin was examined in cells for inhibitory effects on translation and ribosomal-subunit formation. It exhibited concentration dependent inhibitory effects on viable cell numbers, growth rate, protein synthesis and 30S and 50S subunit formation.

In the present study, different concentration of hygromycin was used on sesame tissue for necrosis effect ultimate tissue death. In order to estimate the basal level of the antibiotic concentration, a suitable range of media was prepared and depending upon the number of explants survived. Optimum dose of hygromycin was standardized. Levels of hygromycin at 5.0mg/l were optimized as the minimal inhibitory concentrations in the basal media for screening of regenerant. However few authors used kanamycin as selection marker (Taskin et al., 1999 and Yadav et al., 2010) this may be due to the different gene used by them.
Effect of co-infection and co-cultivation period on plant transformation

Co-infection for 15min of Agrobacterium to the plant cells was attached more to the plant cells and Co-cultivation carried out for 2days. Where investigation carried out by Yadav et al., 2010 took 20-25min for co-infection and 3 days for co-cultivation. Further the explants were inoculated on selection regeneration medium (Medium containing regeneration growth hormones and antibiotics) for production of transgenic plants.

Acetosyringone, a plant phenolic compound naturally secreted by wounded plant cells is known to act as an inducer of virulence (vir) genes of Agrobacterium (Sanyal et al., 2005). Acetosyringone (0-200μM) was utilized. Based on preliminary studies of transient X-Gluc staining at the end of co-cultivation acetosyringone 100 μM was standardized. In the presence of acetosyringone, there was extensive blue coloration in the explants, while in the absence of acetosyringone, X-Gluc staining was comparatively less in sesame explants. The present observation that addition of acetosyringone improved the transient expression efficiency is in agreement with the earlier reports in other plants (Sanyal et al., 2005; Tripathi et al., 2005, Verma et al., 2009; Matsumoto et al., 2009; Induker et al., 2010 Yadav et al, 2010).

Confirmation of gene insertion

Primarily the confirmation of gene insertion was checked by Gus analysis in transgenic cotyledons. Taskin et al., 1999 used callus obtained after transformation process for this analysis and Yadav et al., 2010 have not reported the Gus analysis.
Further confirmation of AP37 gene insertion was done by PCR and finally confirmation was carried out by Southern hybridization analysis.

**GUS gene analysis of transgenic shoots**

A GUS gene isolated from *E. coli* has proven to be very useful in plant transformation studies (Jefferson *et al.*, 1986). Its expression in plant tissues can be very easily detected in a histochemical assay in which a piece of tissue is incubated for several hours in a buffer containing the indigogenic substrate 5-bromo-4-chloro-3-indoyl-1-glucuronide (Jefferson *et al.*, 1986). GUS analysis was carried out with the deembryonated seedling of the transgenic lines of tissue. Shafeay *et al.*, 2011 and Taskin *et al.*, 1999 reported maximum GUS expression in cotyledons, where Yadav *et al.*, 2010 reported maximum in leaves.

**PCR analysis of transgenic plants**

RNA has been isolated from *Sorghum bicolor* which has been converted to CDNA by RT PCR. This CDNA is used as template for isolation of AP37 gene by using gene specific primers and that encodes polypeptides of 243 amino acids and 729bp AP37 is a novel bifunctional enzyme that involved in plant development and stress responses (Oh *et al.*, 2009). PCR analysis for transgenic expression in sesame was carried out by Taskin *et al.*, (1999) and Yadav *et al.*, (2010).

In the present investigation introduction of agronomically important AP37 gene conforming salt and drought resistance has been reported first time in sesame. The same
gene was introduced by other author Oh et al., (2009), Kim and Kim (2009) in *Oryza sativa*.

**Transformation efficiency**

Transformation efficiency through *Agrobacterium tumefaciens* by using deembryonated seed explants were 6 percent. Yadav *et al.*, 2010 reported transformation efficiency was 1.01% in sesame.

**Southern blot analysis of transformed lines**

The original method of blotting was developed by Southern (1975) for detecting fragments in an agarose gel that was complementary to a given RNA or DNA sequence. In this procedure, referred to as Southern blotting, the agarose gel is mounted on a filter paper wick which dips into a reservoir containing buffer. The hybridization membrane is sandwiched between the gel and stack of paper towel which serves to draw the transfer buffer through the gel by capillary action. The DNA molecules are carried out of the gel by buffer flow and immobilized on the nitrocellulose membrane where it is further fixed by UV irradiation. Following the fixation step, the membrane is placed single stranded DNA called the probe (729bp fragment of AP37 gene was used here), which might have complementary, sequences to the blot transferred DNA band to be detected.

The one line of *A. tumefaciens* transgenic line was used for southern blotting analysis. These are PCR conformed positive lines.
Southern blotting analysis is considered to be the most convincing report for the integration of the DNA insert in the plant genome. Digestion of the genomic DNA with *Hind*-III gave rise to the band consisting of the *AP37* fragment from in integrated T-DNA region in the plant genome. Probe of the digested sample of *AP37* gene after transferring on a membrane, showed a distinct positive band at 729bp vicinity.