transformation may comprise manipulation of explant material to embark upon new morphogenetic pathways. It is prerequisite to develop alternative pathway for time consuming *in vitro* regeneration protocols. *In vitro* florigenesis and seed setting is an alternative pathway of plant regeneration[11]. *In vitro* florigenesis has potential to serve as a convenient tool for time-effective studies various aspects of *in vitro* flower bud from initiation to organ development[12]. Keeping this in view, here, we made an attempt to develop an efficient system for *in vitro* florigenesis in Indian soybean genotype bypassing vegetative phases which will be amenable for transgenic development in soybean.

Materials and Methods

Plant material and explant preparation

A popular Indian soybean variety JS-335 representing largest area under cultivation was focused in the present study. Healthy seeds were handpicked, washed several times with sterile water before surface sterilization. The seeds were then surface sterilized with Tween-20 wash, followed by sterilization with 70% ethanol for 30 s and treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 3 min. Sterilized seeds were further rinsed 4-5 times in sterile water to remove traces of $HgCl_2$, blot-dried and plated on disposable Petri dishes containing half MS basal medium and incubated in dark at 25°C for 3 days to germinate seedlings[13]. After that, plates were incubated in light for 2 days. Five days old *in vitro* grown seedlings were used as the source material for further explants preparation.

Preparation of cotyledonary node with axillary bud explants

Two explants were obtained from each seedling through vertical cut of that cotyledonary node with axillary bud (split in the middle of hypocotyls-cotyledon junction) by removing roots and part of hypocotyls approximately 3-5 mm below cotyledonary node. The cotyledons were removed from the seedling and cut vertically using sterile surgical blade.

Culture media and culture condition

MS basal medium supplemented with different concentrations of TDZ (0.5 and 1.0 mg/L) either alone or in combinations with NAA (0.2 and 0.4 mg/L) were tested for *in vitro* flower induction. Explants were transferred to MS basal media having with 0.25 g (w/v) phytagel (HIMEDIA), and fortified with 3 g (w/v) sucrose and supplemented with either singly or in combination of TDZ (0.5 and 1.0 mg/L) with NAA (0.2 and 0.4 mg/L). Each glass bottle containing 25 mL medium was inoculated with 5-6 cotyledonary node with axillary bud explants in such way that the abaxial side was touching the surface and cultures were incubated in light-dark (16-8 h) photoperiodic conditions of cool white-fluorescent light providing a quantum flux density of 60 $\mu mol/m^2/s$ at 25°C. The pH of all media was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. For each treatment, three replicates of 30 explants were taken.

Average percent of *in vitro* flower induction was recorded at different time interval. The average values of all data were tabulated for interference(s). Finally, the medium and PGR combination possessing the best response was selected as the ‘flower induction medium (FIM) and used in all subsequent experiments.

In vitro flower development

The stages of *in vitro* flower development on FIM and time taken thereof was recorded visually and under stero-zoom microscope (Nikon SMZ800, Japan). These were also photographed using digital camera (Samsung EK-GC-100). Stages of single *in vitro* flower bud development at different time interval were recorded. A total 6 treatments were used to study the development of individual flower bud and floral organs from 14-45 days.

Cytological studies

In order to understand the organization of flower bud and individual flower with its floral organ development, flower buds were fixed in a mixture of ethanol, chloroform and acetic acid (6:3:1 w/v) for 24-48 h, rinsed with 70% ethanol and refrigerated until use. The *in vitro* flower buds were finally observed under microscope (SMZ- 800 Nikon, Japan) and photographed using digital camera. Based on the recorded observations a floral diagram and floral formula for *Glycine max* was constructed.

Comparative morphology of *in vitro* and *in vivo* flower buds

The morphology of the *in vitro* and *in vivo* flowers was compared to ascertain their similarity. For this, soybean plants growing *in vivo* were tagged at flowering stage. The *in vitro* flower buds were grouped into five different developmental stages growing on FIM at 14 days interval for 45 days.

In both the *in vitro* and *in vivo flowers*, parameters such as (i) stages of individual flower bud development; (ii) morphological development of individual flower bud; (iii) dissected floral parts; and

(iv) stages of androecium and gynoecium development were recorded. The developmental stages of *in vitro* and *in vivo* flower buds were compared on the basis of their morphological features.

The morphology and the pollen viability from *in vitro* and *in vivo* grown floral buds were also compared. For this, anthers of both types of flower buds were stained through 1% vital dyes *viz.*, methylene blue, neutral dye; and aniline blue using the procedure described by Johri & Vasil[14] and pollen germination as per Rodriguez-Riano & Dafni[15] and observed under the compound microscope. Photographs of pollen viability were captured using digital camera (Samsung EK-GC-100).

Statistical analysis

Data was analysed statistically following factorial complete randomized design (F-CRD). Each treatment was replicated thrice containing 30 explants in each replication. The mean of each treatment and their interactions were compared at probability level (P) of ≤0.05.

Results

Induction of *in vitro* flowering

The cotyledonary node with axillary bud explant expanded at least twice of their original size on the medium augmented with TDZ and NAA within 14 days of culture condition. Flower buds regenerated from proximal end of the explant and developed into flowers synchronously. The treatments used for *in vitro* florigenesis showed significant difference for the flower induction (Fig. 1). The number of flowers per explant varied with plant growth regulator (PGR) combination and concentrations (Table 1). Amongst the PGR combinations tested, the treatment A2B2 enriched with 1.0 mg/L TDZ combined with 0.5 mg/L NAA was proved to be optimal for inducing maximum number of flower buds (60.42%) followed by the treatment A1B2 (33.18%) supplemented with 0.5 mg/L TDZ along with 0.2 mg/L NAA and A2B1 (18.27%) augmented with 1.0 mg/L TDZ without NAA. However, the lowest response of *in vitro* flower induction was recorded in the treatment A2B3 (10.51%) supplemented with 1.0 mg/L TDZ and 0.4 mg/L NAA. Interestingly, the treatment A1B1 comprising MS medium supplemented with 0.5 mg/L TDZ which considered as control treatment showed 23.19% of flower induction.

The cultures with flowers on media containing TDZ resulted into abscission of flowers after 40th days of incubation. Hence, the explant, with or without flowers, were shifted onto MS basal medium during 30-35 days of incubation on medium containing TDZ. The explant cultured on optimal flower regeneration

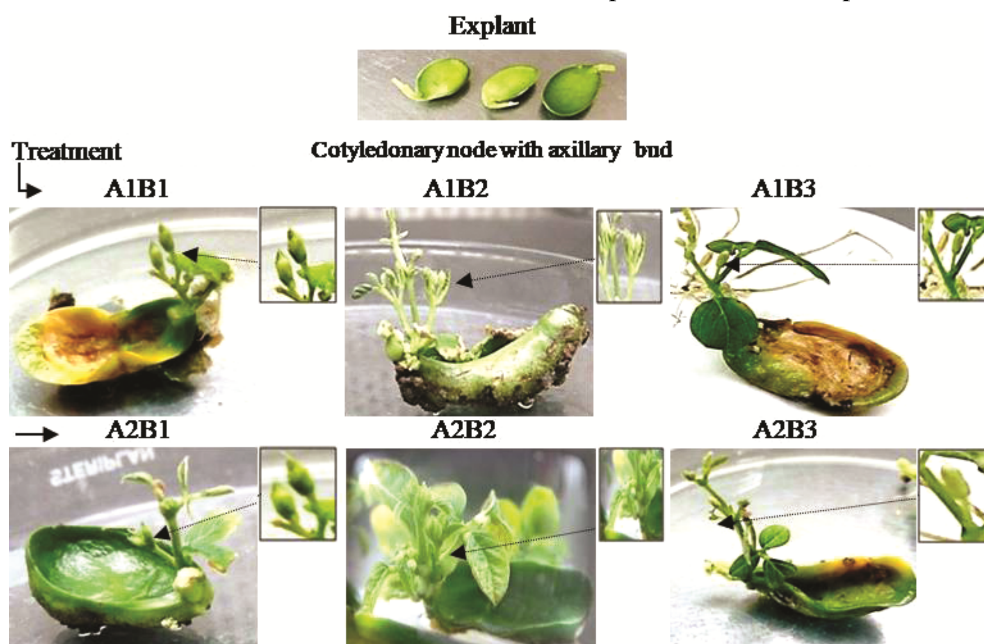

Fig. 1 — *In vitro* flower induction on different treatments of TDZ and NAA combinations (A1B1, A1B2, A1B3, A2B1, A2B2 and A2B3) using cotyledonary node with axillary bud explant of soybean variety JS-335. [All treatments consisting MS basal medium (Murashige and Skoog 1962) supplemented with different concentrations and combinations of TDZ and NAA as (1) A1B1: MS + 0.5 mg/L TDZ + 0.0 mg/L NAA; (2) A1B2: MS + 0.5 mg/L TDZ + 0.2 mg/L NAA; (3) A1B3: MS + 0.5 mg/L TDZ + 0.4 mg/L NAA; (4) A2B1: MS + 1 mg/L TDZ + 0.0 mg/L NAA; (5) A2B2: MS + 1.0 mg/L TDZ + 0.2 mg/L NAA; (6) A2B3: MS + 1.0 mg/L TDZ + 0.4mg/L NAA]

medium supplemented with 1.0 mg/L TDZ with 0.2 mg/L NAA, showed flower regeneration and seed set indicating that the flower organogenesis was strongly influenced by concentration of TDZ.

Average timeline of *in vitro* florigenesis

The physiological difference between *in vitro* flower induction originating from different combinations of TDZ and NAA was influenced by time of flower induction. *In vitro* flower induction was recorded in six treatment combinations of TDZ and NAA at different time intervals (14th, 20-25, 30-35, 40-45 days after inoculation). Average percent of *in vitro* flower induction ranges from 14-45 days as illustrated in Fig. 2. Significant difference was recorded in the in vitro flower induction on 20-25 days after inoculation in all the treatments. Similarly, the significant increase in number of flower bud was recorded up to 30-35 days and subsequently, it starts pod and seed development. The highest flower induction under *in vitro* was initiated during 14-20 days in A2B2 treatment (27.33%) followed by treatment A1B2 (22%). However, only 5% flower induction was recorded on the control treatment A1B1 and 6.33% of flower induction was recorded from treatment A2B1. Plant growth regulator combination of A2B2 was proved to be optimal for *in vitro* flower induction of 49% on 20-25 days followed by treatment A1B2 (22%). The treatments A1B3 and A2B3 were recorded 3.6% and 0.33% *in vitro* flower induction, respectively. During 30-35 days, maximum flower induction of 60.42% was recorded in A2B2 treatment supplemented with 1.0 mg/L TDZ and 0.2 mg/L NAA. Whereas, the A1B2 treatment recorded 33.18% of flower induction followed by 23.19% in the A1B1 treatment (control) supplemented with 0.5 mg/L TDZ. Treatment A2B1 and A1B3 were recorded 18.27 and 16.12% *in vitro* flower induction, respectively. The lowest *in vitro* flowers induction of 10.51% was observed in A2B3 treatment.

Table 1 — Effect of TDZ with NAA on *in vitro* flower induction using cotyledonary node with axillary bud explant

	Treatment	Concentrations of TDZ (Factor A)	
	Concentrations of NAA Factor B)	0.5 mg/L TDZ (A1)	1.0 mg/L TDZ (A2)
	0.0 mg/L NAA (B1)	23.19 (28.79)	18.27 (25.30)
	0.2 mg/L NAA (B2)	33.18 (35.17)	60.42 (51.01)
	0.4 mg/L NAA (B3)	16.12 (23.67)	10.51 (18.92)
	Factor A	Factor B	Factor A×B
Critical difference	2.41	2.09	4.18
SE (d)	1.09	1.34	0.95
SE(m)	0.77	0.95	1.34
	significant at 5% level	significant at 5% level	significant at 5% level

The significant effect of TDZ and NAA was recorded on *in vitro* flower induction at different time intervals. These results suggested that the presence of TDZ in combination of NAA supported *in vitro* florigenesis. Higher concentration of plant growth regulator invariably affected florigenesis in the genotype used. The best response of 60.42% *in vitro* florigenesis was obtained when basal MS medium supplemented with 1.0 mg/L TDZ and 0.2 mg/L NAA, hence that treatment was designated as best "Flower Induction Medium". The Average percent flower induction of flower induction medium (A2B2 treatment) possessed 2-3 fold higher *in vitro* florigenesis than the other treatments tested.

***In vitro* florigenesis on flower induction medium (FIM)**

The average values of *in vitro* florigenesis were tabulated for interference (Table 1) and medium with plant growth regulators (PGR) combination yielding the best response was identified as "Flower Induction Medium (FIM)".

In the present investigation, 1.0 mg/L TDZ with 0.2 mg/L NAA (A2B2) was showed high rate of

Fig. 2 —Average percent of *in vitro* flower induction in soybean genotype, JS-335 on different treatments of TDZ and NAA from cotyledonary node with axillary bud as explants. [All treatments consisting MS basal medium (Murashige and Shoog 1962) supplemented with different concentration and combination of TDZ and NAA as- 1) A1B1: MS + 0.5 mg/L TDZ + 0.0 mg/L NAA; 2) A1B2: MS + 0.5 mg/L TDZ + 0.2 mg/L NAA; 3) A1B3: MS + 0.5 mg/L TDZ + 0.4 mg/L NAA; 4) A2B1: MS + 1 mg/L TDZ + 0.0 mg/L NAA; 5) A2B2: MS + 1.0 mg/L TDZ + 0.2 mg/L NAA; 6) A2B3: MS + 1.0 mg/L TDZ + 0.4 mg/L NAA]

in vitro florigenesis and selected as "flower induction medium". In the *in vitro* florigenesis, different developmental stages of flower and its growth on medium augmented with 1.0 mg/L TDZ in combination of 0.2 mg/L NAA was recorded at different time intervals from 14 to 45 days (Fig. 3). Cotyledons of soybean genotype, JS-335 expanded at least twice their original size on medium augmented with TDZ and NAA within 14 days of culture. Subsequently, deep greenish structure differentiated into flower buds from proximal end of the cotyledonary node with axillary bud explants. *In vitro* flower buds regenerated from cotyledonary node with axillary bud developed into flower synchronously. Initiation of flower buds started at 14-20 days after inoculation. The multiple numbers of flower buds were developed during 20-25 days to 30-35 days. Number of flower per explants was dependent on combination and concentration of plant growth regulator. The medium supplemented with 1.0 mg/L TDZ and 0.2 mg/L NAA was proved to be optimal for inducing maximum number of flower bud per cotyledonary node with axillary bud explants. *In vitro* flower on the medium containing TDZ resulted into flower abscission after 40 days due to ethylene production[16-19]. Hence, explants with flower buds were transferred to basal MS medium for pod development. Regenerated flowers set pods, as a result of self-fertilization. The pods fully matured and turned yellowish-green colour within 45-48 days. Each pod contained one well developed seed. These seeds were germinated when implanted on solidified medium and grown normal plant.

Development of floral bud and its organ development

Proximal end of cotyledonary node of soybean showed *in vitro* flower induction. Five distinct stages of flower development were recorded at different time intervals and depicted in Fig. 4. Flowers were immature at 22-25 days. Androecium comprised of small white colour filament with immature small

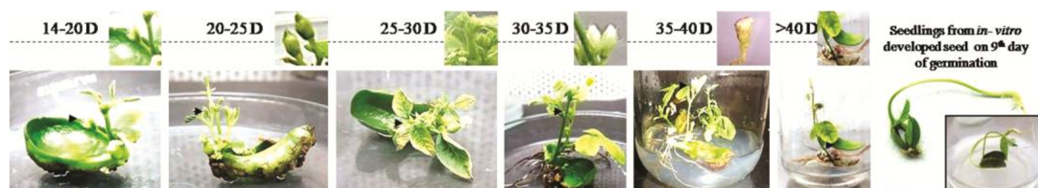

Fig. 3 — *In vitro* flower regeneration in soybean variety, JS-335 on flower induction medium (FIM). [Different stages of flower development on the FIM (A2B2) supplemented with 1.0 mg/L TDZ + 0.2 mg/L NAA (FIM) at different time intervals in D (days)]

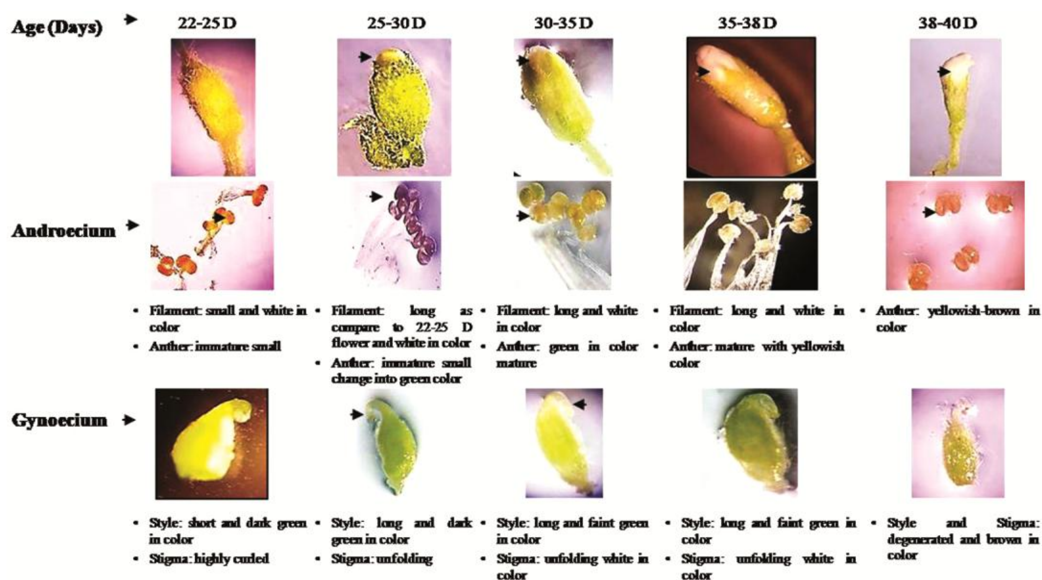

Fig. 4 — Developmental stages of floral organ during *in vitro* florigenesis using soybean variety, JS-335. [22-25 D- Immature flowers, androecium with white filament and small anthers, gynoecium comprised with small green style and curled stigma; 25-30 D- flowers with small opening with floral organs, androecium with long white filament and gynoecium with long and dark green colored style; 30-35D-well developed flowers, androecium with long white filament and slight green anthers, gynoecium with long white filament, slight green style and unfolded white stigma; 35-40 D- matured flower ready to pod development, androecium with yellow brown anthers and degeneration of style and stigma]

anther whereas, gynoecium comprised of short and small style with highly curled stigma. Flower with 22-25 days old were observed to immature and initiated development of floral organs. Small white filaments with immature small anther were observed under keel petals. While, gynoecium with short dark green style and highly curled stigma were observed.

The 25-30 days old flowers were perceived with small opening with development of floral organ. The androecium comprised of long white filament as compare to previous flower. The gynoecium comprised of long style dark green in colour and unfolding was observed in stigma. Flowers were developed with increase in time interval. The 30-35 days old flowers were observed with well-developed floral organs. However, androecium comprised of very long white filament with mature slight green coloured anthers. Gynoecium comprised of long and slight green colour style and white unfolded stigma.

The 35-38 days old flowers were fully matured comprising yellow anther and long white filaments. Pollens of anther were found viable in pollen viability test. Similarly, gynoecium comprised of long and faint green colour style with unfolded white stigma. During 38-40 days after flower induction, flowers were completely matured and ready to pod development. Androecium with yellow brown anthers was observed under microscope. After fertilization, style and stigma were degenerated.

Comparative floral morphology of *in vitro* and *in vivo* grown plants

Both *in vitro* and *in vivo* flowers were organized in zygomorphic symmetry. The *in vivo* flowers were larges as compared to *in vitro.* Soybean flower were bisexual in nature because it contains androecium and gynoecium; and zygomorphic (bilateral) symmetry was found in flower. *In vitro* flowers of soybean were small as compared to *in vivo* flower. In floral biology of *in vitro* flower, calyx was composed of 5 sepals fused to each other. The calyx was relatively large in proportion to flower and was gamosepalous (united sepals) in nature. Corolla consisted of five petals which enclose pistil and ten stamens. Stamens were 10 in number and diadelphous (two bundles) in nature. Nine stamens were developed around pistil and tenth stamen remained free. Flower had large standard petal, two small wing petals and keel petal that enclose the stamina column. All reproductive organs were remaining enclosed in keel petal.

Based on above result, the floral diagram of flower bud was drawn and floral formula for each flower bud was written as “% ⚥K (5), C (1+2+ (2), A (9) +1, G1” where % ⚥ represented zygomorphic symmetry and bisexual plant in nature, respectively; the K (5) denoted calyx with 5 fused sepals; and the C (1+2+2) represented corolla. Corolla was composed of one standard petal, two wing petals and two keel petals. ‘A’ denoted androecium with nine fused anthers and one separated where, the G denoted gynoecium.

***In vitro* florigenesis cycle of soybean variety, JS-335**

Six days old aseptic seedlings were used as source material for explants preparation and cotyledonary node with axillary buds were used as explants. Within 14-16 days, cotyledonary node with axillary bud of soybean genotype, JS-335 were expanded at least twice their original size on media supplemented with TDZ and NAA.

The average timeline of *in vitro* florigenesis cycle was depicted in Fig. 5. One cycle of *in vitro* florigenesis cycle takes time period of about 35-40 days from inoculation to complete *in vitro* flower development. Consequently, pod development was initiated due to self-fertilization. The combination of 1.0 mg/L TDZ with 0.2 mg/L NAA resulted in *in vitro* florigenesis within 35-40 days, with average percent of 60.42% *in vitro* flower induction and seed set development. Above results revealed that *in vitro* florigenesis cycle was completed in 45 days. *In vitro*

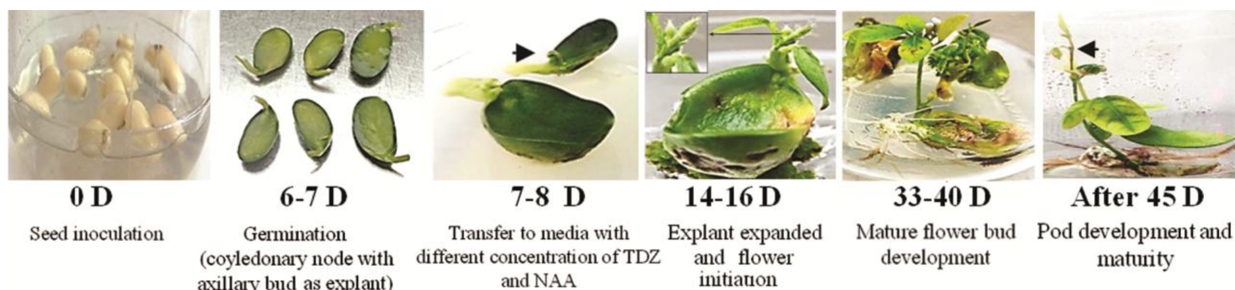

Fig. 5 —Average timeline in days (D) of *in vitro* florigenesis in soybean variety, JS-335 using cotyledonary node with axillary bud explants. [A complete cycle of 35-40 days required for *in vitro* florigenesis using cotyledonary node with axillary bud explant incubated on flower induction medium]

florigenesis was revealed *in vitro* flower induction was strongly influence by concentrations and combinations of TDZ and NAA.

Discussion

Individual plant cell conveys the potential of generating a plant under defined conditions via either organogenesis or embryogenesis. The term '*in vitro* flowering' should not be confused with 'florigen/ florigenesis'. Formation of organ from explant is known as "organogenesis". More precisely, generation of roots and shoots is termed as 'rhizogenesis' and 'caulogenesis', respectively. Here in this pathway, flowers are regenerated from the explant without producing shoots. Hence, it is not unreasonable to term the phenomena of direct flower formation from explants 'florigenesis' (flower organogenesis). The *in vitro* flowering reveals the vegetative meristem of plants produced via organogenesis or embryogenesis is converted into flowering meristem due to physiological or chemical stimuli. Therefore, the phenomenon of direct flower bud formation form explant referred as florigenesis. In the florigenic pathway, flowers regenerated from the explant without producing shoots. Similar results on direct flower bud regeneration were earlier reported in model plant *Nicotiana* using thin layer of pedicel as explants[20].

In the present study, flower regeneration occurred only from the proximal end of cultured cotyledonary node with axillary bud explants. The removal of the axillary bud inhibited formation of new buds and resulted in the production of calli at both ends of the explants which was not able to regenerate shoots. The axillary buds are essential for formation of multiple bud tissues in soybean. Only the cotyledonary node with axillary bud produces 100% regeneration and formation of multiple bud tissues whereas, those without axillary buds produced excess callus. Histologically it has revealed that exogenously applied cytokinin's altered the development of axillary meristems, promoted proliferation of the meristematic cells in the axillary buds and increased the number of bud primordia which originated from the existing axillary meristems[21].

Similarly, high frequency of flower bud induction was observed in the medium supplemented with 1.0 mg/L TDZ with 0.2 mg/L NAA. Usually, cotyledonary node of grain legumes holds high morphogenetic potential at their proximal end[22,23]. The medium supplemented with 1.0 mg/L TDZ with 0.2 mg/L NAA showed highest percent of *in vitro* flower induction (60.42%) as compared to other treatments. Hence, medium was designed as "flower induction medium" (FIM). Results suggested that concentration of TDZ was the critical parameter that determined flower regeneration, as variation in concentration of TDZ level affected flower bud formation. TDZ is considered as potential growth regulator for *in vitro* shoot regeneration and somatic embryogenesis of several crops[24-28]. Recent findings have shown that this PGR can also promote the transition of vegetative meristem into floral meristem, either alone[29-31]or, in combination with NAA[32,33].

The transition of plant from vegetative to reproductive phase is known to involve a series of morphological, physiological, biochemical and molecular changes[34-37]. These changes are actually the manifestation of complex biological events that unfold sequentially in response to certain environmental conditions while facilitating easy tracking of changes associated with floral-transition and flower development in effective time manner[35].

The ability to regenerate flowers and subsequent seed setting in soybean of practical importance in the synchronous development of pod, which is quite asynchronous, often resulting in considerable loss in yield when attempting crosses. *In vitro* florigenesis technique offers reliable contribution to study of molecular basis and hormonal regulation of flowering and the factors controlling the transformation of vegetative meristem into flowering meristem[35]. This *in vitro* florigenesis technique has valuable tool assisting micro propagators to release new species and genotype(s) into market more rapidly. *In vitro* florigenesis offers viable seed set, if combined with transformation, transgenics can develop through short regeneration cycle of about 45 days using variety JS-335.

Though the numerous genes have been identified in soybean, functional genomics is still lagging behind due to the low transformation efficiency. To introduce targeted trait(s) related to flowering, resistance / tolerance to biotic and abiotic stresses and/or tissue specific genes targeted to flowers, the desired product can be obtained using flower organogenesis pathway avoiding time consuming vegetative phase.

Today transgene escape is a great concern in plant genetic engineering, since most of the transgenic plants harbour the antibiotic resistance genes. Selectable markers are only useful in the laboratory to detect transgenic cells and plants using appropriate antibiotics. Beyond the laboratory, these genes

become annoyance to non-transgenic plants and to the ecosystem. Even though different strategies like co-transformation, *cre-lox* system based genetic tool to control site specific recombination events in genomic DNA which are currently used to eliminate marker genes from the transgenic plants. As an alternative to shoot organogenesis and embryogenesis, if *in vitro* seed setting is realistic for genetic transformation, it is possible to obtain transgenic seeds directly from the test tubes within a short time span. If the transgene elimination strategies are combined with flower organogenesis pathway, marker eliminated transformed seeds can be obtained directly in the advanced generation (T1).

Conclusion

The present study demonstrated simple, efficient and highly reproducible system of in vitro florigenesis in a popular Indian soybean variety JS-335. The investigation offers a 'Flower induction medium' where the floral buds directly induced from cotyledonary node with axillary bud resulted in to homorganic seeds through self-pollination within 40-45 days, where it takes 90-95 days under *in vivo*. The *in vitro* florigenesis system offers new avenues contributing towards various studies. One of the important ones is being able to shorten the life cycles of plants; other aims include studying hormonal regulation of plant flowering at molecular level. Similarly, the system of florigenesis and pod development under *in vitro* described here can be exploited for successful recovery of regenerated plantlets of soybean as well. Importantly, it is a critical step towards the development of transformation, forming part of soybean improvement programme.

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Conflict of interest

Authors declare no competing interests.

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