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Research Article

IN VITRO SEED GERMINATION AND SHOOT REGENERATION OF SANDALWOOD (*SANTALUM ALBUM*) FROM HYPOCOTYL

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ABSTRACT

The East Indian Sandalwood, *Santalum album* L., is a tropical, woody and perennial tree. The fragrant heartwood accumulates the sandalwood oil, rich in sesquiterpenoids, costliest among plant products and is reported to have many medicinal properties. Sandalwood is being inducted into International Union for Conservation of Nature (IUCN), Red List of Threatened Species (IUCN, 2006, 2012) as vulnerable.

The challenges associated in the sustainable cultivation of sandalwood are less flowering and fruit setting, seed dormancy, seed viability and cheap availability of seedlings along with other reasons. The present need is mass distribution of seedlings, raising of sandalwood plantations and expanding their distribution range across the country.

It is, therefore, proposed to undertake studies with following objectives: (i) To enhance the seed germination and (ii) To induce shoots on Murashige and Skoog (MS) medium using direct and indirect approach. The seeds of sandalwood were germinated on the half MS basal media, germinated embryos are used to develop shoot on various combinations of M.S. growth medium supplemented with Cytokinine and Auxin growth regulators. Maximum seed germination 47.91% has been obtained on the plain half M.S. basal media.

Keywords: *Auxin, Cytokinines, Sandalwood, Shoot induction.*

INTRODUCTION

Sandalwood (*Santalum album* L; 2*n* = 20) is one of the important tropical tree which is commercially known for its fragrance (Shashidhara *et al.*, 2003). It is member of family *Santalaceae* and being as perfumery material it is commonly known as "Chandan". It is a small- to medium-sized hemiparasitic tree, distributed rather widely in India. Sandalwood is mostly confined to the South Indian states, especially Karnataka, Tamil Nadu and Kerala, and is indigenous to Peninsular India (Srinivasan *et al.*, 1992).

S. album or Indian sandalwood is of great commercial value due to its fragrant heartwood which yields unique oil preferred for perfumeries, cosmetics, medicines and also in incense sticks industries. Sandalwood oil has antipyretic, antiseptic, antiscabietic, and diuretic properties and is also effective in the treatment of bronchitis, cystitis, dysuria, and diseases of the urinary tract. The seeds are used as diuretic, hypotensive, antitumorigenic, antiviral agents, and for treating a number of skin diseases (Kirthikar and Basu, 1987; Desai and Shankaranarayana, 1990).

Globally, with high economic value of sandalwood and its oil, sandalwood wealth in forests are declining due to overharvesting and illegal poaching in natural habitats (Naseer *et al*., 2012). This alarming genetic

erosion condition indicates that there is need to conserve this commercially important tree species. To protect the species, efforts have been made to establish *ex situ* conservation gardens for sandalwood in India (Rao *et al*,. 2011). But the conservation efforts and planning suffer from lack of information on the level and structure of natural genetic variability of sandalwood populations (Rao, 2004). Hence, to examine the existing genetic variability, there is urgent need for systematic variability study in sandalwood.

Somatic embryogenesis has many potential advantages for mass propagation and genetic improvement of hardwood forest trees (Merkle, 1995). The method offers fast multiplication of high value clones for reforestation and provides a means of gene transfer and the production of new plants from transformed cells for the mass production of transgenic trees. An efficient and highly reliable method for the regeneration of plants from explants is essential for sandalwood. This report describes the regeneration of callus and shoots induction from mature zygotic embryos of sandalwood.

MATERIALS AND METHODS

Seed collection and Germination:

The seeds were collected from rural areas of Latur district from 15-20 years old elite tree. The seeds were black in color when fully matured. The seeds collected were given various treatments to check the germination percent and viability of the seed. Firstly the seeds were soaked in deionized water for overnight at room temperature to check the viability of the seeds (CC Baskin, JM Baskin 1998). The seeds floating at the surface of the water were discarded while the seeds settled at the bottom were selected. Then seeds were treated by dividing it into two groups.

a) Seeds without seed coat b) Seeds with seed coat.

In the seeds without seed coat the pericarp was removed and the seed coat was cracked and seed were removed. Then the seeds were treated giving GA_3 treatment at 0.05% by soaking the seeds for a period of 6 hours and without GA_3 as control (Das, 2013). The seeds were then surface sterilized with 2% fungicide for 10 min followed by stirring and then washed 2x times with distilled water, then seeds were sterilized with 1% sodium hypochlorite (2-3 mins for seeds without seed coat, 5 min for the seeds with seed coat) and then washed three times with distilled water, followed by treatment with 70% alcohol for 2-3 minutes, and then treated with 1% $HgCl_2$ for eight minutes and washed with sterile distilled water in the laminar air flow. Seeds were dried in laminar air flow for 10 mins prior to inoculation (Rai, 2002). Seeds were kept for germination on half strength MS basal media in complete dark room at 25 ^{0}C for a week. The germinated embryo consists of short plumule and thick radicle (Rai, 2002). For each treatment 200 seeds were inoculated.

Regeneration from hypocotyls:

The germinated embryos were excised from the endosperm was inoculated on the MS media supplemented with various combinations, viz., concentration of 2, 4-D, Cytokinins, Auxins, GA_3 and 3% sucrose. The media was solidified with 0.8% agar and pH was adjusted to 5.8 to 6 with 1N NaOH and 1N HCl after adding the growth regulators, and before autoclaving (Murashige and Skoog, 1962)0. The cultures were incubated at 25 ^{0}C under a cool fluorescent light. Each treatment consisted of three explants.

RESULT

Seed Germination:

As the germination percentage of sandalwood seed is very poor and difficult to induce due to presence of dormancy or impermeable hard seed coat, the seed germination obtained in *in-vitro* study is 11.67% (Solle, and Semiarti, 2016). In the present study the seeds were inoculated in two groups i.e. with seed coat and without seed coat and then treated with and without GA_3 to check the germination percent. The results are shown in table 1 given below.

Table 1: Results for seed germination with and without seed coat treated with GA3

Treatments*	Seed Treatment	% Seed Germination	
	Days	**10**	**20**
T1	**With GA_3 (0.05%)**		
	Seeds without seed coat	**37.5**	**----**
	Seeds with Seed coat	**0**	**0**
T2	**Without GA_3 (control)**		
	Seeds without seed coat	**47.97**	**-----**
	Seeds with seed coat.	**0**	**0**

**Each treatment consisted of 200 seeds.*

Figure 1: Shows the procedure for seed treatment and inoculation. A: the seeds collected from rural areas of Latur district. B: seeds soaked in water overnight. C: Seed coat of the seed was removed. D: Seeds with and without seed coat. E: chemicals used for surface sterilization of the seeds. F: seeds inoculated on ½ strength MS media.

Regeneration from hypocotyl:

The germinating seed embryo gave rise to hypocotyl and radicle, the hypocotyl was excised and about 10 mm in length pieces and were horizontally incubated on full strength MS media supplemented with 2,4-D, auxin, Cytokinins and GA3. The above combination and concentrations resulted in the callus induction and shoot regeneration from the mature embryo.

Callus Induction: In the present study the above combinations and concentrations showed the results of callus growth (indirect embryogenesis) as shown in Table 2, and shoot induction (direct embryogenesis) as results shown in Table 3 below. On the media containing MS + 2, 4-D with Kinetin and TDZ alone showed callus formation. The callus formed were fragile and green in color on the media containing 2, 4-D and kinetin, while pale cream in colour and had fragile translucent texture on the media containing TDZ.

Table 2: Showing the results for callus induction

Treatment number	Treatments	Observation (Days)		
		10	20	30
T1	MS basal media (Control)	Increase in Embryo size	Increase in embryo size	No growth observed.
T2	M.S.+ 2.5 mg/l 2,4 D + 3 mg/l Kinetin	Increase in the embryo size	Cottony mass formation.	Cottony mass formation.
T3	M.S.+2mg/l 2,4 D + 2.5 mg/l Kinetin	Increase in the embryo size	Cottony mass formation.	Cottony mass formation.
T4	M.S.+ 3 mg/l 2.4 D + 2.5 mg/l Kinetin	Increase in the embryo size	Cottony mass formation.	Dense cottony mass formation.
T5	M.S.+ 1.5 mg/l 2,4 D + 2 mg/l TDZ	Increase in the embryo size	Cottony mass formation	Dense cottony mass formation.
T6	M.S. + 2µM Kinetin + 0.5µM IAA	No growth observed	Increase in the embryo size	Increase in the embryo size
T7	M.S. + 4µM Kinetin + 0.5µM IAA	Increase in the embryo size	Greenish mass formation	Greenish mass formation
T8	M.S. + 6µM Kinetin + 0.5µM IAA	Increase in the embryo size	Greenish mass formation	Greenish mass formation
T9	M.S. + 8µM Kinetin + 0.5µM IAA	Increase in the embryo size	Greenish mass formation	Dense greenish mass formation
T10	M.S.+ 1.5µM TDZ	Increase in the embryo size	Cottony mass formation	Dense pale cottony mass formation
T11	M.S.+ 2.5µM TDZ	Increase in the embryo size	Cottony mass formation	Dense pale cottony mass formation
T12	M.S. + 2µM TDZ + 0.25µM GA3	No growth observed	Increase in the embryo size	No growth observed
T13	M.S.+ 2µM TDZ + 0.5µM GA3	No growth observed	Increase in the embryo size	No growth observed
T14	M.S. + 4µM TDZ + 1.5 µM GA3	Increase in the embryo size	Cottony mass formation	Dense pale cottony mass formation

**Each treatment consisted of 3 explants*

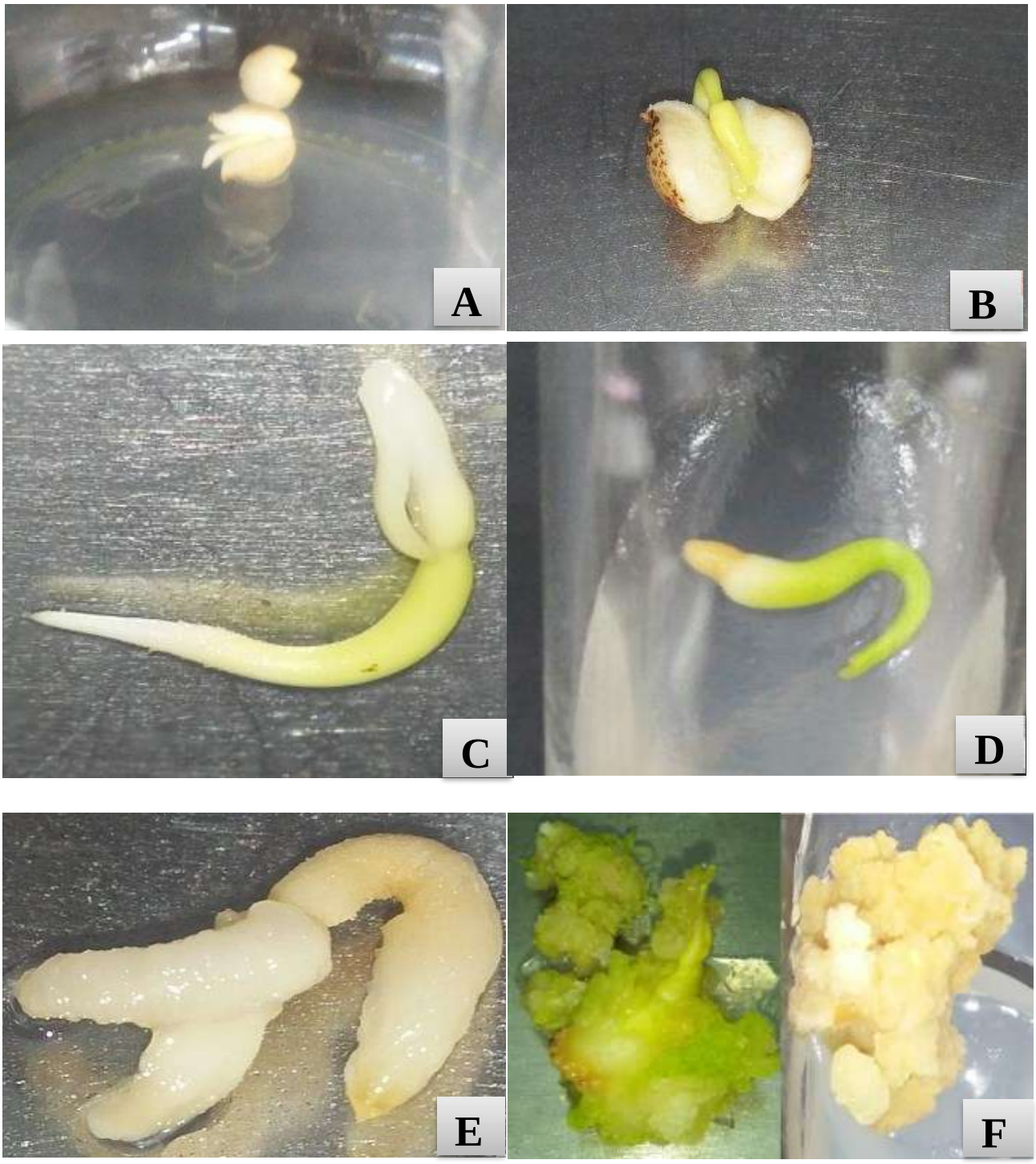

Figure 2: Callus induction in *Santalum album.* A: swollen embryo and endosperm, B: Germinating embryo, endosperm. C: hypocotyl along with radicle excised from the endosperm. D: hypocotyl along with radicle inoculated on MS media. E-F: Callus induction, having translucent texture.

Shoot Induction:

In the present study germinated embryos excised from endosperm were also inoculated on MS media supplemented with various combinations and concentrations of Auxin and GA3.

Table 3: Shows the result for shoot induction

*Treatments	Treatments detail	Observation Table (Days)		
		20	40	60
T1	MS basal media (Control)	No growth observed	Increase in embryo size	Increase in embryo size no growth observed
T2	M.S. + 2µM IAA + 0.25µM GA3	No growth observed	No growth observed	No growth observed
T3	M.S. + 2µM IAA + 0.5µM GA3	No growth observed	Increase in embryo size	Small shoot like growth Observed.
T4	M.S. + 3µM IAA + 0.5µM GA3	No growth observed	Increase in embryo size	Dense formation of root hairs on radicle part.
T5	M.S. + 4µM IAA + 1µM GA3	No growth observed	Increase in embryo size	Bunchy Shoot growth.
T6	M.S. + 6µM IAA + 1.5µM GA3	No growth observed	Increase in embryo size	Shoot Regeneration observed.

**Each treatment consisted of 3 explants.*

DISCUSSION

This study has firstly shown that *Santalum album* can produce shoot without intervening callus phase. As seed germination of S*antalum album* is poor both in *in vivo* and *in vitro* due to presence of dormancy. Clarke and Doran (2012) have stated that no exact cause has been identified for the dormancy of *S. album* and speculated that the seeds of this species might have an exogenous kind of dormancy. However, Baskin and Baskin (1998) inferred that the seeds of this species have physiological dormancy (PD) or perhaps morphophysiological dormancy (MPD), i.e. seeds have a minute embryo that must elongate inside the seed before, during or after the loss of PD. For seed germination *in vitro* the MS half strength media supplemented with BAP 2 mg/l was best media for seed germination with the germination percent of 11.67% (Solle and Semiarti, 2016), in the present study the seeds were germinated on MS half strength media after giving the treatment with and without GA3 by soaking the seeds in water prior to germination to check the germination percent, the maximum percent of seed germination obtained was 47.97%. It has also been noted that when the seeds were surface sterilized and kept for drying in laminar air flow prior to inoculation reduces the rate of contamination (Rai, 2002).

It has been reported that 10-20% of explants produced callus in media containing MS media with BAP and then sub culturing with other hormones was needed to produce embryogenic callus (Lakshmi Sita *et al.*, 1979; Rao and Bapat, 1995). In the present study kinetin along with IAA or TDZ alone spontaneously produced callus with greater reproducibility. The system described here required different concentration and combination as those previously reported to get callus induction and germination. The major problem for propagation of sandalwood through embryogenesis has been the low conversion of somatic embryos

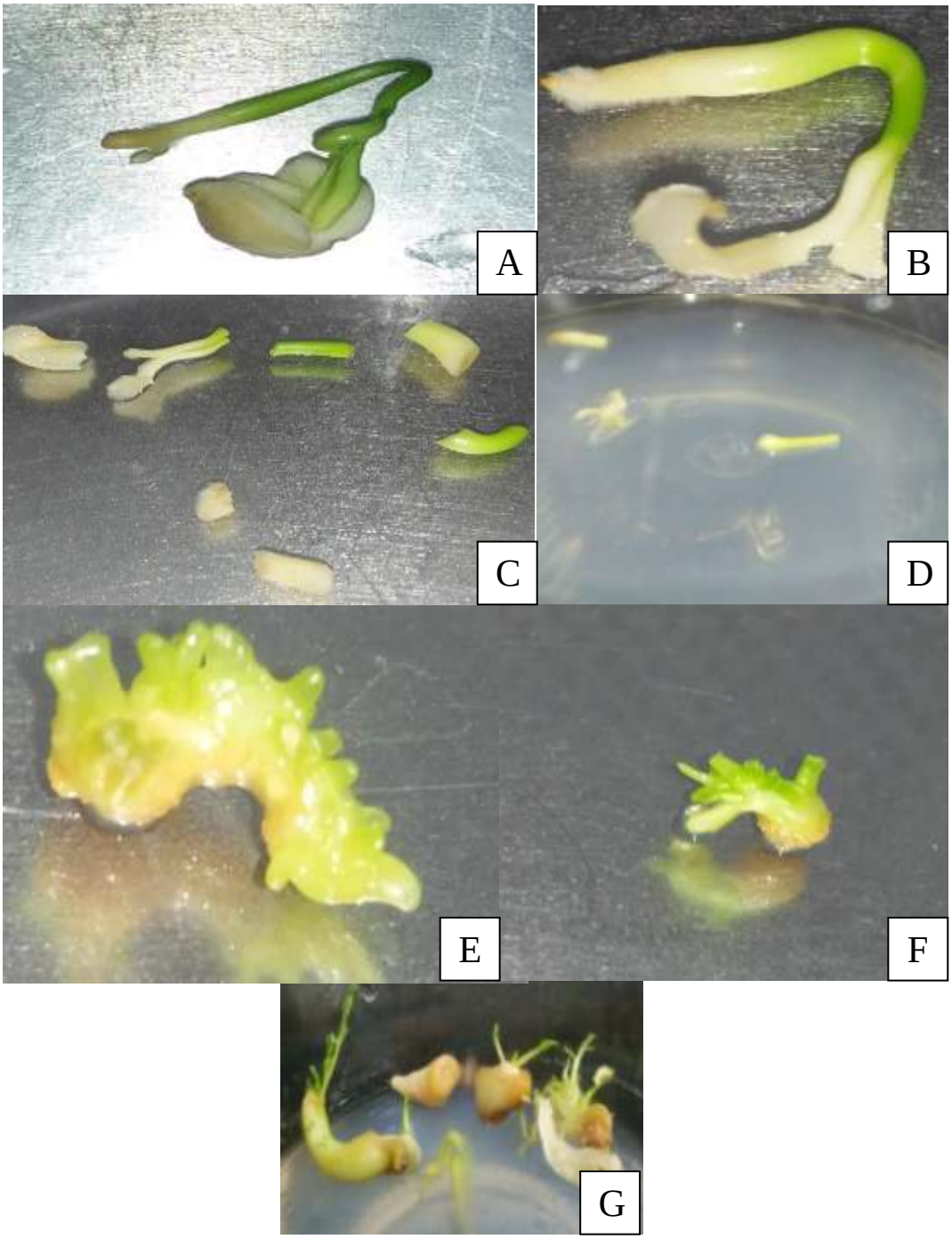

Figure 3: Shoot regeneration in *Santalum album*. A: Fully germinated seed. B: germinated embryo excised from the endosperm. C: Embryo cut into several parts i.e. hypocotyl, and the radicle. D: The cut parts inoculated on MS media. E, F, G: Shoot regeneration on the hypocotyl part of the embryo.

into plantlets (Rao and Bapat, 1995). Rao and Bapat (1995) reported that plantlets were obtained from only 30% of cultured embryos. The media containing IAA and BAP resulted in to low conversion and multiplication of somatic embryos in to plantlets (Rao and Bapat 1995). In the present study the germinated embryos excised from the endosperm were cut into several parts such as radicle, hypocotyl, and the plumule part and were inoculated on the MS media supplemented with various combination of Auxin, Cytokinins and GA3. After inoculation the several parts of embryo resulted in swelling and increase in size. The MS media supplemented with IAA and GA3 proved beneficial for the induction of shoots from hypocotyl part of the embryo within 8 weeks.

In sandalwood the optimum concentration requirement of Auxin, Cytokinins, and GA3 for callus and shoot induction was low as compared to other species. In the present study the kinetin 2-8µM/l along with IAA 0.5µM/l and TDZ 1-2.5 µM/l proved optimum concentration for callus induction, while IAA ranging from 1-6 µM/l and GA3 0.2- 1.5 µM/l proved optimum concentration for shoots regeneration.

CONCLUSION

In the present study the GA3 did not showed enhanced seed germination, however, without seed coat treatment showed enhanced seed germination (47.97 %) over control.

The callus induction was observed on media containing 2-4 D, Kinetin along with IAA, and TDZ alone at various combination and concentration.

M.S. + 3 mg/L 2,4-D + 2.5 mg/L Kinetin,
M.S. + 8µM Kinetin + 0.5µM IAA,
M.S. + 2µM TDZ + 0.25µM GA3.

The shoot induction was observed **at concentration of IAA ranging from 2µM to 6µM and GA3 ranging from 0.5 µM to 1.5µM.**

M.S. + 6 µM IAA + 1.5 µM GA3 proving the best combination for shoot regeneration.

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