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A rapid *Agrobacterium*-mediated transformation protocol for tomato (*Solanum lycopersicum* L.) cv. PKM-1

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Abstract
Factors that influence transformation efficiency such as age of explants, plant growth regulator concentration, antibiotic concentration, *Agrobacterium* density, and infection time were optimized with a view to establish a high-throughput transformation protocol for tomato (*Solanum lycopersicum* L.) cv. PKM-1. Seedling explants were transformed using a binary vector carrying *nptII* gene, conferring kanamycin resistance by *Agrobacterium* mediated transformation. Transformation efficiency of 27 % and 17 % respectively was observed when cotyledon and hypocotyl explants were collected from 7 and 9 day old seedlings, co-cultivated with an *Agrobacterium* suspension of $O.D_{600}$ of 0.4 and 0.2 for 30 and 10 minutes infection time respectively. Better shoot development was observed when shoots induced on cotyledonary and hypocotyl explants were cultured on modified MS medium containing 0.5 and 1 mg/L zeatin respectively. Supplementation of medium with 200 mg/L timentin effectively suppressed *Agrobacterium* overgrowth. Furthermore, the time required from inoculation of explants with *Agrobacterium* to transfer of transgenic tomato plants to soil was only 70 days as compared to 3 to 4 months in standard tomato transformation protocols.

Keywords: Tomato transformation, cotyledon, optical density, infection time, regeneration

Introduction
Tomato (*S. lycopersicum L.*) belonging to *Solanaceae*, is one of the economically important horticultural crops. It has achieved remarkable acceptance and admiration in recent years with the discovery of its bioactive compounds such as lycopene which possesses anti-oxidative activities and anti-cancer functions (Wu *et al.* 2011; Raiola *et al.* 2014) [29, 22]. Thus, tomato production and consumption are constantly increasing.

Tomato offers several characteristics that enable studies on plant development, fruit ripening and investigation of many plant-pathogen interactions that affect economically important plants. The enormous amount of researches engrossed on tomato allowed the development of new biotechnological tools and platforms for genetic and genomic analyses (Barone e*t al.*, 2008) [1] such as virus-free plant production (Moghaieb *et al.,* 2004) [18], genetic transformation (Ling *et al.,* 1989) [14], which allows the introduction of foreign genes into a germplasm, without modifying the genetic background of popular varieties.

Since the first published reports on *Agrobacterium*-mediated transformation and regeneration of cultivated tomato using leaf disc by McCormick *et al.,* (1986) [15] was made, several reports on optimization of different factors which influence tomato transformation have been published from several laboratories across the globe. Some of the considered factors that influence regeneration include genotype (Sharma *et al.*,2009; Madhu and Savithramma, 2014, Jamous and Abu-Qaoud, 2015) [25, 16, 11], type of explants (Bhatia *et al.,* 2005; Rajesh *et al.* 2016) [2, 23], plant growth regulators (Gubis *et al.*, 2003, Namitha and Negi, 2013) [9, 20] and antibiotics used (Briza *et al.*,2008; Mamidala and Nanna, 2009) [3, 17]. Nonetheless, transformation and regeneration of tomato can still be considered difficult based on the widely variable rates of success achieved. *Agrobacterium*-mediated transformation has been the commonly employed method to transfer a foreign gene into the genome of plants and has been the technique with highest rate of success. However, in tomato, high transformation rate has been confined to model cultivars, such as Moneymaker (van Roekel *et al.,* 1993) [27] and Micro-Tom (Cruz-Mendı´vil, 2011) [5].

In order to achieve high and efficient transformation rates for different cultivars, a standardized protocol must be established by considering the factors which greatly influence *Agrobacterium*-mediated genetic transformation such as the type and age of the plant material, *Agrobacterium* strain and bacterial density; infection time, duration of co-cultivation, several physiological and biochemical conditions of *Agrobacterium* infection, and the selection regime (Li *et al.*, 2013) [13]. In view of this, we made an attempt to establish an efficient transformation protocol system for a cultivar PKM-1 by optimizing some of the afore-mentioned parameters such as type and age of explant, *Agrobacterium* cell density, infection time, concentration of timentin to control *Agrobacterium* overgrowth and concentration of zeatin for shoot development.

Materials and Methods

Preparation of Explants

Genetically pure seeds of tomato cv. PKM1 seeds were obtained from Horticultural College and Research Institute, Tamil Nadu Agricultural University, Periyakulam**,** Tamil Nadu. Tomato seeds were pre-soaked in water containing a few drops of tween 20 for five min and subjected to vigorous shaking for another 5 min. Seeds were treated with seventy per cent ethanol for 4-5 min, followed by washing with sterile water thrice. The seeds were then treated with 4 per cent sodium hypochlorite for 7 min with occasional swirling followed by doubled distilled water wash thrice. The seeds were blot dried on a sterile tissue paper and placed on half strength MS medium (Murashige and Skoog, 1962) [19] for germination under 72 hours dark period followed by a cycle of 16 hours photoperiod using cool white fluorescent tube light (110-130 nM/m^2/s intensity) and eight hours of darkness at 26˚C in a plant growth chamber (Panasonic, Japan).

Type and age of explant

The influence of type and age of explant for regeneration on MS medium modified with Gamborg vitamins (Ruturaj *et al.*, 2014) **[24]** and supplemented with 1.0 mg/L zeatin was evaluated. Cotyledon and hypocotyl explants were collected from 7, 8, 9, 10 and 12 day-old *in vitro* grown seedlings. In case of cotyledon, distal and proximal ends (1-2 mm) were cut off and the explants were cut into two pieces before placing them on the pre-culture medium. They were handled gently with flat forceps to avoid any injury. Cotyledonary explants were placed in such a way that the abaxial side was in direct contact with medium. In case of hypocotyls, they were cut into pieces of 1.0 cm and then placed on the medium. These explants were pre-cultured for a day under light prior to co-cultivation.

Bacterial strain and plasmid

Tomato hypocotyls and cotyledons were co-cultivated with *Agrobacterium* strain LBA4404 harbouring a binary vector pCAMBIA2300, with neomycin phosphotransferase (*nptII*) gene driven by *CaMV35S* promoter, conferring resistance to kanamycin which was used for plant selection.

Bacterial density and infection time for transformation

A single colony of *Agrobacterium* LBA4404 (pCAMBIA 2300) was inoculated into 3 mL Luria Bertani (LB) medium amended with kanamycin 50 mg/L, tetracycline 10 mg/L and rifampicin 20 mg/L and allowed to grow overnight in an incubator shaker at 28°C and 180 rpm. From the overnight culture, 500 µl was sub-cultured into 30 ml LB with same antibiotics and conditions and grown for 6-8 hours. The pellets were harvested by centrifugation at 4,000 rpm for 10 min. The pellet was washed with 30 ml of MS broth and suspended in MS broth containing 100 mM acetosyringone. *Agrobacterium* cultures at 0.1, 0.2, 0.3, 0.4 or 0.5 OD at 600 nm were used for co-cultivation. The pre-cultured explants were carefully submerged in the suspension (30 ml of co-cultivation suspension) for 10, 20, and 30 minutes. Post infection, explants were blotted on sterile tissue paper and transferred on to co-cultivation medium. Plates were kept under dark condition in a growth chamber for 48 hours.

Concentration of timentin for control of *Agrobacterium* overgrowth

After co-cultivation, the infected cotyledons and hypocotyls were transferred on to shoot regeneration medium (MS-B5 medium containing 1 mg/L zeatin) supplemented with different levels of timentin (0, 100, 200, 300, 400 mg/L). They were sub-cultured onto a fresh medium at 15 days intervals.

Concentration of zeatin for shoot development

The effect of different concentration of zeatin (0.5, 1, 1.5 and 2.0) on shoot development was studied. The regenerating shoots were sub-cultured every 15 days onto same fresh medium until they are about 1 cm long. Thereafter, they were sub-cultured on selection medium supplemented with above-mentioned concentration of zeatin. Regenerated shoots were maintained under selection medium with antibiotics. They were maintained in same media composition until they reached 2-3 cm long before transferring into rooting medium.

Rooting, hardening and acclimatization of plantlets

Elongated shoots (2-3 cm) were transferred to the half MS basic medium, supplemented with IBA (1 mg/L) for rooting and maintained under 16 hours light and 8 hours dark. Shoots with no sign of rooting after 21 days were discarded. Well rooted plants were hardened in greenhouse in small cups containing autoclaved coconut peat mixture covered with a polythene cover to maintain humidity. Well-established plants were transplanted into bigger pots and maintained in transgenic greenhouse.

Molecular confirmation of the transformants

To confirm the presence of the *nptII* gene in the putative transgenic plants, plant genomic total DNA was isolated following CTAB (cetyltrimethyl ammonium bromide) protocol,
which is a modification of the method of Doyle and Doyle (1987) [7] from both transformed and non-transformed (i.e. negative control) plant samples and used as templates for PCR. Plasmid DNA (pCAMBIA 2300) was used as a positive control.

Result and Discussion

Though a number of earlier reports suggest availability of efficient protocols for regeneration and transformation of tomato, the frequency of regeneration and transformation depends on tomato genotype (Park *et al*, 2003) [21] and there is no single protocol applicable for all cultivars (Sun *et al.*, 2015) [26]. In the present study, we have attempted to optimize a few key factors that influence shoot regeneration and transformation in a popular cultivar of tomato, PKM-1. The factors which we considered include type and age of explant,

Agrobacterium culture density, infection time and concentration of timentin.

Type and age of explant

The response of hypocotyl and cotyledonary explants collected from *in vitro* grown seedlings of different age (7 to 12 days) on MS medium supplemented with 1 mg/L zeatin is presented in Table 1. Seven day old cotyledonary explants and nine day old hypocotyl explants exhibited better response in terms of regeneration of well-developed shoots. Poor response was observed with older explants. In our study, cotyledons of all ages showed callus initiation response (100%) after 10 days of culture (data not shown) but only a few developed into shoots. However, hypocotyl explants showed direct shoot regeneration from the cut ends (Fig. 1-A and D) after 13-15 days of culture. The least response in both explants was observed with 12 day-old explant indicating that regeneration is not favoured in aged explant. Himabindu *et al.*, 2012 [10] reported that cotyledons and hypocotyls collected from 10 day-old seedlings were found to be superior compared to explants collected from 8, 12, 14 day-old seedlings. Furthermore, Famiani *et al.*, (1994) [8] proposed that explants from young plants show more regeneration potential than older ones as the younger leaves are still developing, have less differentiated and more metabolically active cells, and therefore, under suitable hormonal and nutritional conditions show improved plant regeneration.

Table 1: Effect of explant age on regeneration of tomato cv. PKM-1.

Explant	Age (days)	No of explant	Percentage of Responding Explant	Percentage of explant with elongated shoots
Cotyledon	7	60	81.67 (65.0)	75.00 (59.0)
	8	60	71.67 (52.8)	61.67 (46.0)
	9	60	73.33 (57.9)	55.00 (49.8)
	10	60	48.33 (45.9)	28.33 (34.2)
	12	60	38.33 (41.2)	16.67 (26.6)
SEd			4.01	3.42
C.D (0.05)			7.89	7.30
Hypocotyl	7	60	43.33 (44.1)	28.33 (34.2)
	8	60	66.67 (54.8)	48.33 (46.9)
	9	60	81.67 (65.9)	71.67 (60.2)
	10	60	76.67 (59.0)	58.33(51.8)
	12	60	21.67 (28.9)	13.33 (21.3)
SEd			2.85	2.26
C.D (0.05)			5.02	4.83

Figures in parentheses are transformed values

Concentration of *Agrobacterium* optical density and infection time

The effect of *Agrobacterium* culture density and duration of infection on tomato transformation efficiency was studied and presented in Table 2. The highest transformation efficiency (27 %) was observed in cotyledons when they were infected with *Agrobacterium* at $O.D_{600}$ of 0.4 for 30 min while 13 % transformation efficiency was observed in hypocotyls infected with culture at $O.D_{600}$ of 0.2 for 10 min.

Table 2: Effect of bacterial concentration and infection time on transformation of Tomato cv. PKM-1.

Explant	Culture density	Infection time (min)	Percentage of explants showing shoot initiation	Percentage of shoots responded for rooting	Transformation efficiency (%)
Cotyledon	0.2	10	47 (43.1)	7 (12.60)	0.00 (0.91)
	0.2	20	47 (43.1)	13 (21.15)	0.00 (0.91)
	0.2	30	43 (41.1)	10 (18.44)	6.67 (12.60)
	0.4	10	43 (41.1)	17 (23.86)	6.67 (12.60)
	0.4	20	73 (59.2)	40 (39.15)	16.67 (26.99)
	0.4	30	90 (75.0)	53 (46.92)	26.67 (31.00)
	0.6	10	40 (39.2)	10 (18.44)	3.33 (6.75)
	0.6	20	27 (31.0)	3 (6.75)	0.00 (0.91)
	0.6	30	23 (28.8)	3 (6.75)	0.00 (0.91)
SEd			5.46	5.42	5.05
C.D (0.05)			11.47	11.40	10.61
Hypocotyl	0.2	10	87 (72.3)	47 (43.08)	16.67 (26.99)
	0.2	20	73 (59.0)	27 (31.00)	6.67 (12.60)
	0.2	30	37 (36.9)	7 (12.60)	0.00(0.91)
	0.4	10	67 (55.0)	27 (31.00)	3.33 (6.75)
	0.4	20	50 (45.0)	10 (18.44)	0.00(0.91)
	0.4	30	13 (21.2)	0 (0.91)	0.00 (0.91)
	0.6	10	27 (31.0)	7 (12.60)	0.00 (0.91)
	0.6	20	27 (30.8)	0 (0.91)	0.00 (0.91)
	0.6	30	23 (28.8)	0 (0.91)	0.00 (0.91)
SEd			6.37	4.26	4.29
C.D (0.05)			13.39	8.96	9.02

Figures in parentheses are arc sin transformed values. Transformation efficiency = no. of explants producing transgenic plants/total no. of explants co-cultivated X 100

Concentration of timentin for controlling *Agrobacterium* overgrowth

One of the major problems in tomato transformation experiments is *Agrobacterium* overgrowth on explants and the developing shoot. Earlier experiments conducted in our laboratory showed that the antibiotic, cefotaxime was not effective in controlling overgrowth of *Agrobacterium* on cocultivated explants and developing shoots. Cefotaxime and carbanicillin are the most commonly used antibiotics in genetic transformation experiments. However these antibiotics have shown a great negative impact on regeneration of transformed explants of tomato (Costa *et al*, 2000) [4]. Timentin is another antibiotic used for growth suppression of *Agrobacterium* for many plants (Leamkhang and Chatchawankanphanich, 2005) [12]. It is a semi-synthetic antibiotic widely used to inhibit a broad spectrum of bacterial activity against gram-positive, gram-negative, aerobic and anaerobic bacteria. Timentin (Tim) a mixture of tricarcillin and clavulanic acid and has also been reported to suppress *A. tumefaciens* in tomato genetic transformation experiments (Costa *et al.*, 2000) [4]. In the present study, we have investigated the efficiency of different levels of timentin in controlling *Agrobacterium* overgrowth. There was 100 per cent *Agrobacterium* over-growth when both hypocotyls and cotyledons were cultured on medium without timentin. While in cotyledon, over-growth was observed on 3.33 per cent of the explant on medium containing timentine at 100 mg/L. Contamination was not observed in other treatment in both cotyledons and hypocotyls. Explant survival was observed in all the concentrations of timentin tested. There was no *Agrobacterium* overgrowth on explants or shoots regenerated on medium with 200-400 mg/L of timentin. We did not observe any adverse effect on shoots at 400 mg/L. Earlier reports suggest a decrease in explant survival at higher concentration of cefotaxime and carbanicillin (Mamidala and Nanna, 2009) [17]. Leamkhang and Chatchawankanphanich, (2005) [12] found that 300 mg/L of timentin did not suppress the *Agrobacterium* completely and it required weekly subculture to prevent complete overgrowth. But in our work, 200 mg/L was sufficient to suppress the growth of *Agrobacterium*. Additionally, we observed that there was a noticeable increase in the induction of multiple shoots per explant (data not shown).

Optimization of zeatin concentration for shoot development

The effect of various concentrations of zeatin (0.5, 1.0, 1.5, and 2.0) on shoot development from both cotyledon and hypocotyl explants of PKM-1 was studied and reported in Table 3. Based on the pre-existing protocol in our laboratory, zeatin at 1 mg/L was used for both shoot induction and development. However, at 1 mg/L zeatin, shoot induction was observed but without noticeable development. Shoots initiated on 1 mg/L zeatin were sub-cultured onto different concentrations of zeatin. The highest response was observed in cotyledons and hypocotyls with a mean value of 8.5±0.5 and 7.0 ± 1.1 at 0.5 and 1 mg/L zeatin respectively. The regeneration response of tomato to plant growth regulators (PGRs) has been highly genotype-specific (Wayase and Shitole, 2014) [28], and as such, the type and concentration appropriate for one genotype may be unfavorable for others and our study also shows that the response varies with type of explant.

Table 3: Effect of zeatin on shoot development of Tomato cv. PKM-1.

Explant	Zeatin (mg/ L)	Number of elongated shoots (Mean ± SE)
Cotyledon	0.5	8.5 ±0.5
	1.0	6.5 ±0.5
	1.5	5.0 ±0.1
	2.0	3.5 ±0.5
SEd		0.039
C.D (0.05)		0.086
Hypocotyl	0.5	1.0 ±0.1
	1.0	7.0 ±1.1
	1.5	4.5 ±0.4
	2.0	0
SEd		0.051
C.D (0.05)		0.111

Confirmation of Transformants

In order to confirm the status of putative transgenic plants generated in our study, total genomic DNA was isolated from the leaves of putative transgenic plants and PCR assay was carried out using *nptII* specific primers. The expected amplification size of 430 bp was observed in the test samples as compared to the control, confirming the presence of transgene (Fig. 2).

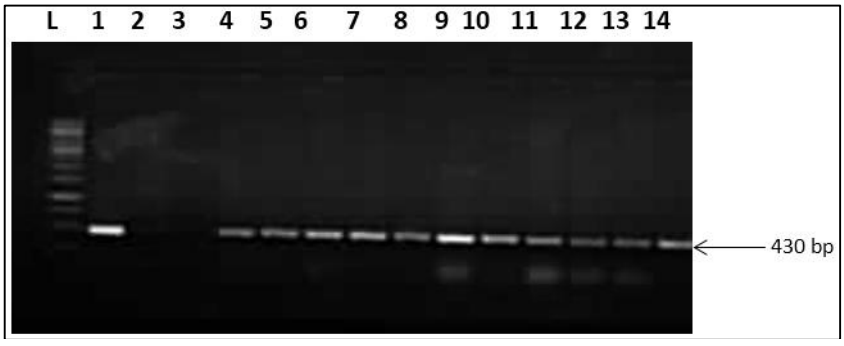

Fig 2: Screening of putative transformants of tomato by PCR.

Lane L: 1000 bp ladder, Lane 1: Plasmid control, Lane 2: Non-transformed plant, Lane 3: Water control; Lane 4-14: Putative transformants

Apart from transformation frequency, transformation cycle (that's from beginning to end of tomato transformation) is another crucial constraint in evaluating the transformation efficiency. Sun *et al.*, 2015 [26] reported a cycle of 120 days, Dan *et al*., 2006 [6] reported 90 days and we report a cycle of 70-75 days with the use of only zeatin as a plant growth regulator in the regeneration medium. A complete transformation cycle in our protocol (Figure 3) only needs approximately 90 d, including explants preparation (7 d), co-cultivation (2 d), shoot induction (30 d), shoot proliferation (15 d) and rooting (20 d). The main reason for this short transformation cycle was the direct transgenic shoot induction from the explants and not from the callus.

To conclude, through the present investigation, a standardized regeneration and transformation protocol was developed for the local tomato variety cv. PKM1 using *Agrobacterium* mediated transformation system. *A* transformation efficiency of 27 % and 17 % respectively was observed when cotyledon and hypocotyl explant were collected from 7 and 9 day old seedlings, co-cultivated with an *Agrobacterium* suspension of $O.D_{600}$ of 0.4 and 0.2 for 30 and 10 minutes infection time respectively. Well-developed shoots were observed when shoots induced on cotyledonary and hypocotyl explants were cultured on modified MS medium containing 0.5 and 1 mg/L zeatin respectively. Furthermore, our results indicated a reduction in transformation cycle which saves time, energy and consumables and may contribute to insure the genetic stability sought in many applications of genetic engineering.

Fig 1: *Agrobacterium* mediated transformation of tomato cv PKM-1 (a) Cotyledonary explants on pre-culture medium (b) Hypocotyl explants on pre-culture medium (c) Co-cultivated explants on selection medium at 3rd selection (D) Hypocotyl explants on selection medium at 1st selection (E) Cotyledonary explants at 3rd selection (F) Hypocotyl explants at 2nd selection on selection medium (G) Elongated shoot on rooting medium (H) Well established transformants in transgenic greenhouse.

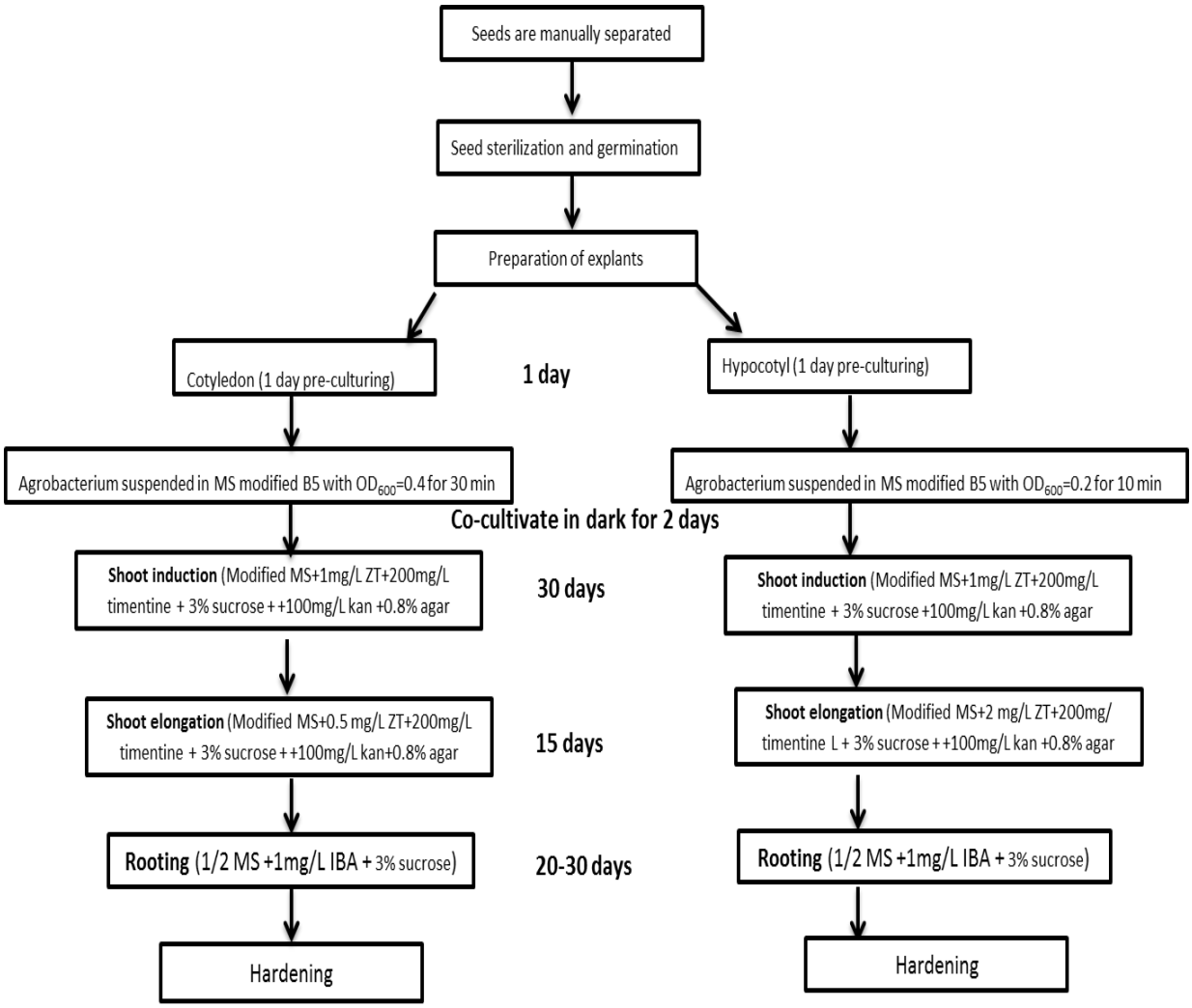

Fig 3: Protocol used for *A. tumefaciens*-mediated tomato (cv. PKM-1) transformation.

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