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Response of tomato in diverse antibiotic selection media for shoot regeneration studies

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Abstract
Tomato (*Solanum lycopersicum* L.) is an important vegetable crop cultivated throughout India. A good *in vitro* plant regeneration system for mass propagation of high-quality and disease-resistant tomato via genetic engineering is required for further improvement of the commercially important cultivars. *In vitro* plant regeneration was assessed in tomato (*Solanum lycopersicum.cv.* PKM1) using cotyledon and hypocotyl explants from seven to eight days old aseptically grown seedlings. Shoot initiation was achieved by culturing cotyledon and hypocotyl explants on Murashige and Skoog (MS) medium supplemented with different concentrations of kanamycin and hygromycin. The effects of two antibiotics namely hygromycin and kanamycin, on the shoot regeneration of co-cultivated explants of PKM1variety cultured on MS medium with phytohormones, were studied. The ability of tomato cotyledon and hypocotyl explants to regenerate into entire plants via direct organogenesis was tested and drying of explants was observed after two weeks of subculture in all concentrations under both antibiotics. Shoot initiation was started after two weeks of subculture in kanamycin ata concentration of100mg/land three weeks of subculture in hygromycin at 10mg/l.

Keywords: Kanamycin, hygromycin, *in vitro* regeneration, *Agrobacterium tumefaciens*, cotyledon, hypocotyl

1. Introduction
Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family and is one of the most economically important horticultural crops. It originated in South America, Mexico, and Central America, and in recent years, it has attained extraordinary acceptance. With the discovery of its bioactive constituents in recent years, such as lycopene, which has anti-oxidative and anti-cancer properties (Raiola *et al.*, 2014; Wu *et al.*,2011) [25, 30], tomato production and consumption are constantly increasing. It is grown worldwide in fields, greenhouses, and net houses and is very versatile to grow either for fresh fruits or processing. It ranks third among vegetable crops (next to potatoes and sweet potatoes) with an annual production of 187 million metric tonnes in the year 2020 (FAOSTAT, 2022). Additionally, this plant is a prototype for introducing important agronomic genes into dicotyledonous crop plants (Paduchuri *et al.*,2010) [20].

In vitro regeneration of cultivated tomatoes has been a subject of research because of the commercial value of the crop and its amenability for further improvement via genetic manipulation. A successful genetic engineering system that aims to use genetically modified plants for commercial purposes needs a good *in vitro* regeneration system. Addressing both basic and applied research, problems will be made easier with the successful connection of a regeneration system with gene transfer techniques (Bhatia *et al*., 2004) [1].

An effective regeneration strategy is essential for genetic engineering that aims to improve plants. The use of particular antibiotics, such as selectable or germicidal ones, is necessary for procedures regularly utilized (agro infection) to introduce foreign genes into a plant genome (Gerszberg A and Grzegorczyk-Karolak., 2019; Kazemi *et al.*, 2014; Sun *et al.*, 2015) [9, 13, 29]. Several factors *viz*., species, genotype, explant type, and culture conditions were involved in the selection of co-cultivated tissue in antibiotic medium and the concentration of selection agent to be added into the selection media also depends on the antibiotic used. (Farzaneh*et al.*, 2013; Gerszberg*et al.*, 2015b; Gerszberg A and Grzegorczyk-Karolak.,2019; Mamidala and Swamy Nanna, 2009; Sun *et al.*, 2015) [4, 6, 9, 16, 29].

The establishment of a regeneration protocol is a prerequisite for genetic transformation. The genotype, regeneration ability of explants, explant position in the medium, and also the impact of antibioticson explants affect tomato plant regeneration (Mamidala and Nanna, 2011) [21].

The preparation of explants, inoculation of explants with *Agrobacterium* cells, co-cultivation, selection of transformed cells from the majority of the non-transformed cells, and regeneration of transformed cells are all steps in the *Agrobacterium*-mediated transformation process that results in a fully transformed plant. Several factors involved in genetic transformation greatly influence the overall gene transfer efficiency (Hu and Phillips 2001; Rai *et al.*, 2013) [11, 23]. The present study was conducted to assess the cultivar PKM-1 for plant regeneration from hypocotyls and cotyledon leaf explants under different concentrations of antibiotic selection.

2. Materials and Methods

Seeds of tomato *cv*. PKM1 was obtained from Horticulture College and Research Institute, TNAU, Coimbatore for tomato transformation.

2.1 *In vitro* germination

Seeds were treated with sterile water and a few drops of tween 20 by vigorous shaking for 3- 5 min. and treated with 70% ethanol for 5min, followed by sterilization with 4%sodium hypochlorite along with a few drops of tween 20 for 7 min with intermittent swirling followed by half strength rinsing with sterile water for 4-5 times. The seeds were blot dried on a sterile tissue paper and placed in a half-strength MS medium (Murashige and Skoog, 1962) for germination in dark for 3-4 days 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 up to 8-10 days.

2.2 Preculture

Eight to ten days old seedlings grown in half-strength MS medium are taken for preculture and both cotyledons and hypocotyl are used for the study. In the case of cotyledon, distal and proximal ends (1-2 mm) were cut 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 the medium. In the case of hypocotyls, they were cut into pieces of 1.0 cm and then placed on the preculture medium containing MS+B5 vitamins (4.4g/L) supplemented with 1mg/L Zeatin and incubated for 48 hours at 26 °C in 16h light and 8h dark.

2.3 Antibiotic sensitivity assay

Explants are treated with kanamycin and hygromycin in concentrations ranging from 50 mg/L to 150 mg/L and 5 mg/L to 10 mg/L, respectively. The cotyledon and hypocotyl that are seven and eight days old are placed on a preculture medium. After two or three days of preculture, explants are moved to kanamycin media. Based on the response of explants, kanamycin and hygromycin concentrations were fixed.

2.4 Co-cultivation

One day before cocultivation, a single colony of *Agrobacterium* (LBA4404: pKSE401-*eIF4E1*and pRGEB31-*eIF4E1*) was inoculated into 3ml LRTK broth (LB medium with kanamycin-50mg/L, rifampicin-10mg/L, tetracycline-5mg/L) and incubated overnight at 28°C in 180rpm. From overnight grown culture, 600µL was taken and inoculated into fresh 30ml LRTK broth and placed at 28 °C for 5 to 6 hours. The *Agrobacterium* was harvested by centrifuging the culture at 5000rpm for 5 minutes and resuspending the pellet with half-strength MS broth and adding to the Petri plate which contains ½ MS broth and 100µM ACS and half-strength MS broth containing 100µM ACS was taken separately, then cell suspension was dissolved. The pre cultured explants were immersed in *Agrobacterium* cell suspension for 15-20min, followed by blot drying using sterile tissue paper. Once after drying, leaf bits were transferred to Whatman filter paper which is placed above the cocultivation medium containing preculture medium supplemented with 250mg cefotaxime. The Petri dish was then incubated at 26 °C for 48 hours in dark conditions.

2.5 Selection and regeneration

After 48 hours, co-cultivated leaf bits were washed with ½ MS broth and cefotaxime (250mg/L) for 5 minutes and blot dried on sterile tissue paper, and placed on a selection medium supplemented with Zeatin (1mg/L), cefotaxime (250mg/L) kanamycin (100mg/L) for pKSE401 construct and Hygromycin (10mg/L) for pRGEB31. At every 15 days interval, the explants were sub-cultured and transferred onto a fresh selection medium. The plates were kept in a plant growth chamber at a temperature of 26°C, 16:8 (light-dark).

2.6 *In vitro* rooting

Elongated shoots (2-3 cm) developed from hypocotyl and cotyledon explants were excised and transferred onto an MS medium fortified with 1.0 mg/L IBA, kanamycin-100mg/L, hygromycin (10mg/L), and cefotaxime-250mg/L. maintained under 16 hours light and 8 hours

3. Results

3.1 Response of explants in different antibiotic conditions

Transformation frequencies in *Agrobacterium*-mediated tomato transformations are related to the explants, such as the seedling age and pre-cultivation time, in addition to the *Agrobacterium* density, co-cultivation time, and infection time. After 2 weeks of culture on different concentrations of kanamycin, there was no inhibition of shoot initiation in 50mg/L and 75mg/L, and drying symptoms were observed in all concentrations of kanamycin and in a medium containing 100mg/L of kanamycinone or two explants showed shoot initiation after 4 weeks of subculture. In higher concentrations, there was no shoot initiation (Table1). Similarly, five days old explants that are placed in a preculture are transferred to different concentrations of hygromycin from 5mg/L to 10mg/L. After 6 weeks of subculture complete inhibition of shoot initiation was observed in 10mg/L of hygromycin (Table2).As compared to kanamycin selection, explants showed less response and also the time taken for shoot initiation was more in the hygromycin selection.

3.2 Regeneration efficiency in kanamycin and hygromycin selection medium: The hypocotyl and cotyledonary explants was co-cultivated with *Agrobacterium* strain LBA4404 harbouring (pKSE401-eIF4E1&pRGEB31-eIF4E1) for 48hours. Regeneration frequency was observed in the range of 12.24% - 37.83% in cotyledon and 4.1%-18.0% in hypocotyl in kanamycin selection (Table3) whereas in

hygromycin selection it recorded 6.94%-31.06% in cotyledon and 2.02%- 14.54% in hypocotyl respectively. (Table 4).

$$\text{Regeneration frequency} = \frac{\text{No of plants regenerated}}{\text{No. of explants cocultivated}} \times 100$$

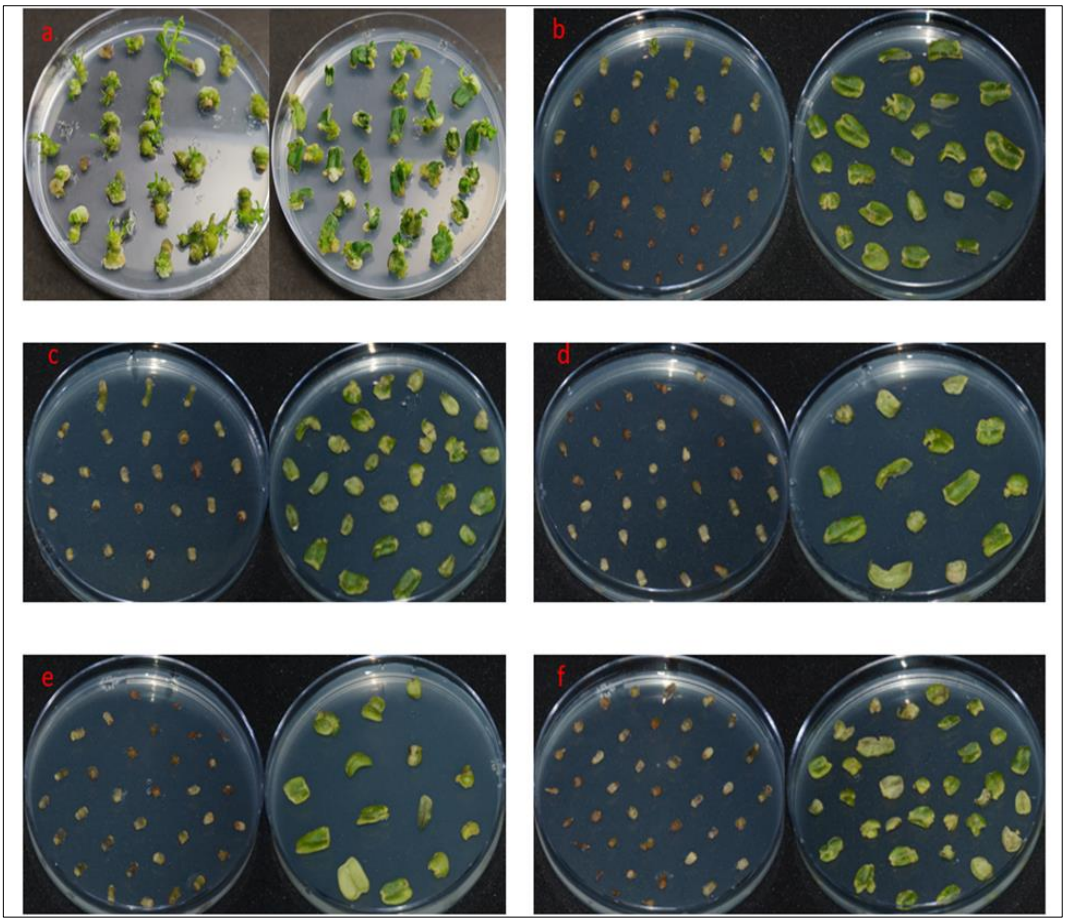

Fig 1: Kanamycin sensitivity test of tomato (*Solanum lycopersicum* cv PKM1) by using cotyledons and hypocotyls a)control without antibiotic b) explants treated with 50mg/L of kanamycin c) explants treated with 75mg/L of kanamycin d) explants treated with 100mg/L of kanamycin e) explants treated with 125mg/L of kanamycin f) explants treated with 150mg/L of kanamycin

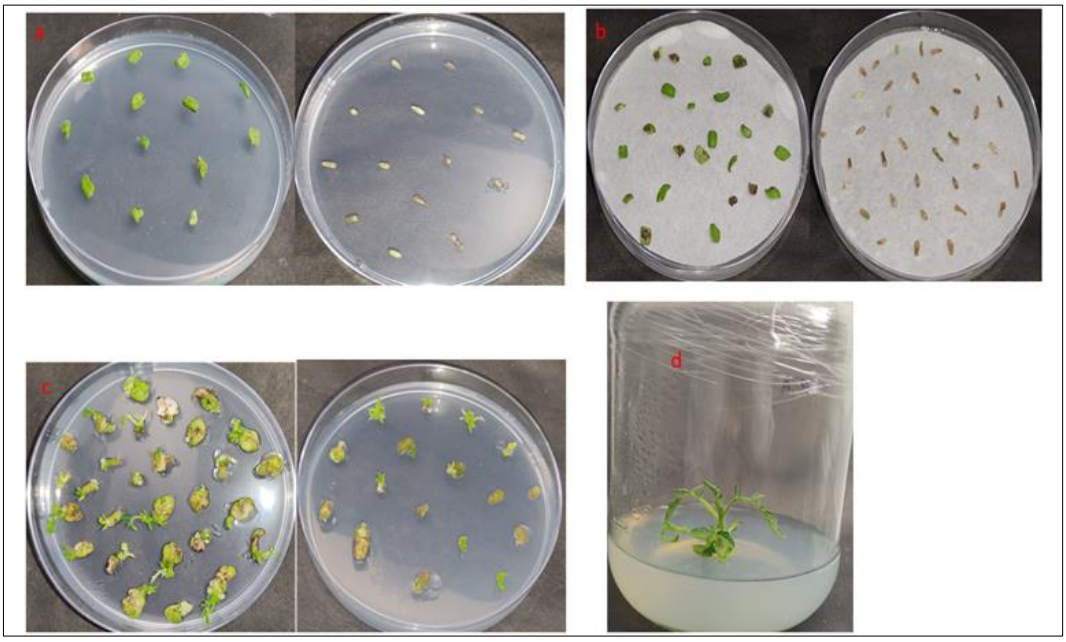

Fig 2: Agrobacterium-mediated transformation of tomato with pKSE401-eIF4E1 construct a) cotyledon and hypocotyl on preculture medium b) cocultivation of cotyledon and hypocotyl c) shooting in cotyledon and hypocotyl d) regenerated shoot transferred to rooting

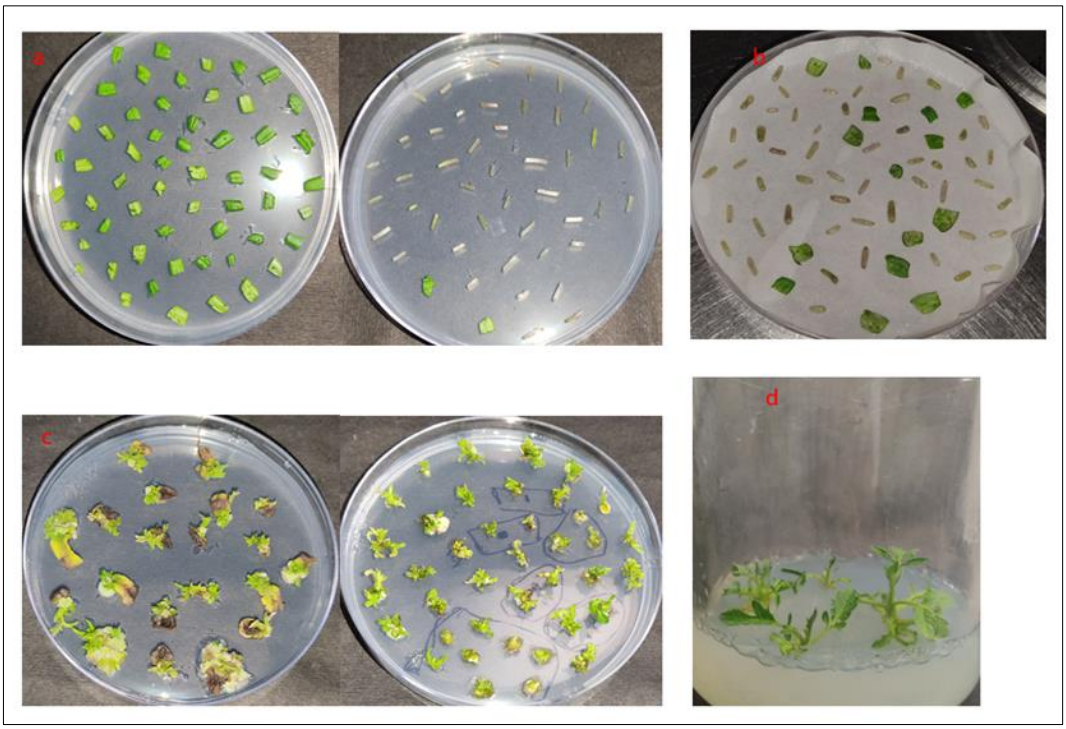

Fig 3: Agrobacterium-mediated transformation of tomato with pRGEB31-eIF4E1 construct a) cotyledon and hypocotyl on preculture medium b) cocultivation of cotyledon and hypocotyl c) shooting in cotyledon and hypocotyl d) regenerated shoot transferred to rooting

Table 1: Kanamycin sensitivity of cotyledon and hypocotyl on the second day from preculture

Kanamycin concentration (mg/l)	Total no of explants inoculated		No. of explants dried after 2 weeks		No. of explants with shoots after 4 weeks	
	Cotyledon	hypocotyl	Cotyledon	hypocotyl	Cotyledon	hypocotyl
Control	50	45	2	5	34	25
Kan50	45	42	8	12	16	6
Kan75	48	46	13	19	7	4
Kan100	47	45	17	21	2	1
Kan125	45	45	21	23	0	0
Kan150	45	45	24	25	0	0

Table 2: Hygromycin sensitivity of cotyledon and hypocotyl on the fifth day from preculture

Hygromycin concentration	Total no of explants inoculated		No. of explants dried after 2 weeks		No. of explants with shoots after 6 weeks	
	Cotyledon	hypocotyl	Cotyledon	hypocotyl	Cotyledon	hypocotyl
Hgy5	4	32	1	10	3	20
Hgy8	8	14	2	3	4	10
Hgy9	10	35	4	17	2	8
Hgy10	5	33	3	19	0	0

Table 3: Agrobacterium-mediated transformation of tomato with pKSE401-eIF4E1 construct

S. No.	No. of Explants Cultivated		No. of Explants dried in Selection after 2 weeks		No. of explants with shoot initiation after 4 weeks		Total No. of explants with shoots (in rooting)		Regeneration frequency (%)	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
1	114	191	5	17	19	15	19	19	13.19	9.94
2	99	145	7	29	21	20	23	25	23.23	17.24
3	138	195	16	48	24	6	44	8	31.88	4.10
4	116	175	13	25	26	13	40	23	34.48	13.14
5	74	100	11	37	19	6	28	18	37.83	18.0
6	115	148	5	35	23	11	30	17	26.08	11.48
7	40	45	9	30	8	5	12	8	30.0	17.77
8	109	153	28	54	18	9	27	12	25.23	7.84
9	49	83	8	24	4	1	6	5	12.24	6.02
10	14	13	1	6	2	0	5	2	35.71	15.38

Table 4: Agrobacterium-mediated transformation of tomato with pRGEB31-eIF4E1 construct

S. No.	No. of Explants Cultivated		No. of Explants dried in Selection after 2 weeks		No. of explants with shoot initiation after 4 weeks		Total No. of explants with shoots (in rooting)		Regeneration frequency (%)	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
1	132	220	3	20	15	6	41	20	31.06	9.09
2	146	241	4	19	13	3	40	11	27.39	4.56
3	80	117	10	48	6	2	13	5	16.25	4.27
4	110	182	58	102	9	0	23	15	20.09	8.24
5	30	55	5	20	11	2	10	8	30.0	14.54
6	144	198	9	25	5	1	10	4	6.94	2.02
7	106	180	5	21	7	1	15	14	14.15	7.77

Table 5: Comparison of regeneration frequency in kanamycin and hygromycin selection.

Construct Name	No. of Explants Cultivated	No. of Explants dried in Selection after 2 weeks	No. of explants with shoot initiation after 4 weeks	Total No. of explants with shoots (in rooting)	Regeneration frequency (%)
pKSE401-*eIF4E1*	2116	408	250	371	17.53
pKSE401-*eIF4E1*	1941	349	81	229	11.79

4. Discussion

Tomato (*Solanum lycopersicum* L.), a processing vegetable of high biological values, is one of the most studied higher plant species because of its several advantages for genetic, molecular, and physiological studies (McCormick *et al.*, 1986) [17]. But reliable callus induction and regeneration of viable plants is considered a limiting step to the successful use of modern techniques in the genetic improvement of major crops (Murphy 2003) [19]. Thus, in order to establish a competent genetic transformation system in the cultivar, it is necessary to test the plant regeneration efficiency from cotyledon and hypocotyl explants for experimental use.

Antibiotics are frequently employed as a selective agent or to eradicate *Agrobacterium* in culture media for plant tissue (Gerszberg, 2018) [8]. Antibiotics added to culture media have been shown to affect morphogenetic processes in *in vitro* cultures either favorably or unfavorably (Davood*et al.*, 2016; Gerszberg A and Grzegorczyk-Karolak., 2019; Grzebelus and Skop, 2014; Meng *et al.*, 2014; Saporta *et al.*, 2014) [2, 9, 10, 18, 28].

In vitro germinated seedlings are used as explants source for shoot initiation which further depends on the culture medium. Seven to eight days old cotyledons and hypocotyl are used as an explants source. Shoot initiation was reported within 2 weeks on the cut surface of both explants on a medium containing kanamycin and in the case of a medium with hygromycin shoot initiation was reported within 3 weeks. In the case of hygromycin, callus formation was observed, due to this it is taking more time for shoot initiation and multiple shoots are also more. Cotyledons are more effective than hypocotyl in both kanamycin and hygromycin selection.

The range of kanamycin, an effective amino glycosidic antibiotic, exploited as a successful selective agent of transformed tomato plantlets is between 50 and 100 mg/l (Kaur and Bansal, 2010; Li *et al.*,2015; Ma J *et al.*, 2015; Rai *et al.*, 2013; Riggs *et al.*, 2001) [12, 14, 15, 23]. In our study, kanamycin at a concentration of 125mg/l and 150mg/l totally prevented regeneration from untransformed explants of cotyledon and hypocotyls, and in 50mg/l, and 75mg/lof kanamycin concentration some explants showed shoot initiation.

Initially, transient gene expression was chosen based on hygromycin resistance. According to the type of explant, transformation trials showed significant variability in the frequency of transformation (cotyledon or hypocotyl). Cotyledonary explants were found to be more effective for regeneration and subsequent transformation than hypocotyl explants. Similar observations were reported by Gadir*et al.*, (2017) [5]. The obtained results showed that kanamycin (50mg/l and 75mg/l) and hygromycin (5mg/l, 8mg/l, and 9mg/l) at lower concentrations will not affect the shoot regeneration. Sandhya *et al.* (2022) [27] used MS medium supplemented with 2.0 mg l^{-1} ZEA and 0.1 mg l^{-1} IAA, 20 mg/l hygromycin for the selection of explants of a variety Arka Vikas. Delayed selection of co-cultivated explants after 15 days followed by continuous selection using 10 mg/l hygromycin improved shoot regeneration in cultivar Rio Grande (Prihatna *et al.* 2019) [22]. The available reports revealed that the concentration of selection agent used for selection depends on the cultivar used and the regeneration protocol optimized for the cultivar.

5. Conclusion

Plant species and cultivars have a significant impact on how antibiotics affect regeneration. Additionally, the type of antibiotic chosen and its concentration are important considerations because the presence of antibiotics in the medium may prevent a plant from regenerating. Our research supports the notion that antibiotic type, concentration, and cultivar all have an impact on tomato regeneration. It is evident that determining the proper dose and explant type is essential for plant's future transformation and regeneration.

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