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Role of explants collecting seasons and sterilization process on *in vitro* regeneration of guava: A technology hurdle

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Abstract
To standardize the season for explants collection and sterilization protocol for getting success in micropropagation of guava cultivars because excessive exudation of phenols from cut ends caused lethal browning of explant and culture medium. Newly grown, young, disease free and fresh twigs were collected from well grown mother plants and selected as a source of explants in summer season (May-June) and winter season (Oct-Nov and Jan-Feb). Shoot tip and nodal explants (2-3 cm) were sterilized using different chemicals *viz*. citric acid, ascorbic acid, NaOCl, $HgCl_2$, KCl, bavistin and streptomycin etc. The explants collected in April-May showed minimum browning in Hisar Safeda and Lucknow-49. In shoot tip and nodal explants of guava, the minimum contamination was noted under treatment consisted of 0.2% each of bavistin and streptomycin (30 min) followed by 1% NaOCl (5 min.), 0.1% $HgCl_2$ (3 min), 1% KCl (3 min) and 70% Ethanol (30 sec). Results obtained from the study concluded that for successful *in vitro* regeneration, especially for difficult and recalcitrant tree species such as guava, is mainly dependent on the source and season of explants collection and the sterilization protocol.

Keywords: Guava, explants, sterilization, seasons graphical abstract

Introduction
Guava (*Psidium guajava* L.) an important fruit tree of the tropical and subtropical climate is a recalcitrant species with high phenolic exudation in which an efficient *in vitro* regeneration and establishment was very difficult due to phenolic exudation (Singh and Singh, 2018). Guava is well known as "apple of tropics" and its easy cultivation, high nutritional value and popularity of processed products makes it an important produce in international trade (Raj kumar *et al*., 2016; Pereira *et al*., 2017). But, now a day's guava production is seriously hampered by wilting, root-knot nematodes and lack of cultivars (Rai *et al*., 2010) [15].
To overcome these problems, microprogation (rapid process for development of identical offspring) is the only possible solution because traditional methods of breeding are limited due to long juvenile growth periods, and heterozygosity. In this technique, choice of explants, season of explants collection and sterilization plays a very important role in achieving successful establishment of *in vitro* cultures. Judicious selection and use of juvenile material as explants could play crucial role for eventual success in micropropagation. Literature reported that shoot tips or axillary buds have been successfully used for *in vitro* regeneration of woody plants particularly guava (Amin and Jaiswal, 1987; Papudatou *et al*., 1990; Meghwal *et al*., 2010; Bisen *et al*., 2014) [1, 14, 12, 4]. Thus, we tried to standardize the season for explants collection and sterilization protocol for getting success in micropropagation of guava cultivars because excessive exudation of phenols from cut ends caused lethal browning of explant. Most of the explants died within 2 days of inoculation in culture medium within 12-24 hrs of inoculation. Therefore, the study was conducted on four guava cultivars *viz*. Hisar Safeda, Hisar Surkha, Lucknow-40 and Allahabad Safeda to develop strategies to overcome the harmful effects of browning either through neutralization or through the avoidance of toxic substances in the medium.

Material and Methods
The present investigation was conducted to standardize source and seasonal variation of explants, and sterilization protocol for successful regeneration in guava in the Plant tissue culture laboratory of the MBB&B Department, CCS HAU, and Hisar. The plant material was selected and obtained from mother plants (Fig.1; 5-7 year old) of Hisar Surkha, Lucknow-49,

Hisar Safeda, Allahabad Safeda, grown in Farm Research Area, CCS HAU, Hisar. The mother plants were timely pruned for emergence of new shoots. The disease free and well grown mother plants were selected as a source of explant in summer season (May-June) and winter season (Oct-Nov and Jan-Feb). Newly grown, young and fresh twigs were collected from the mother plants of four cultivars of guava (Fig. 1).

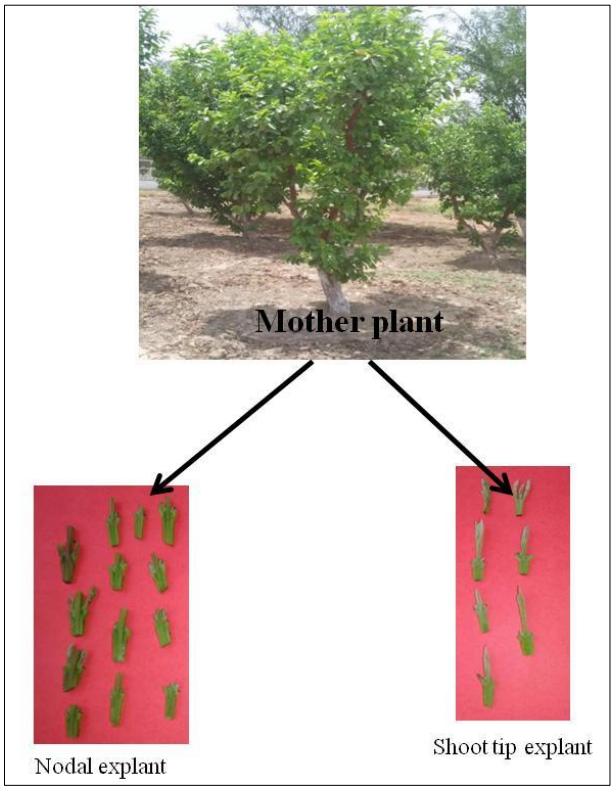
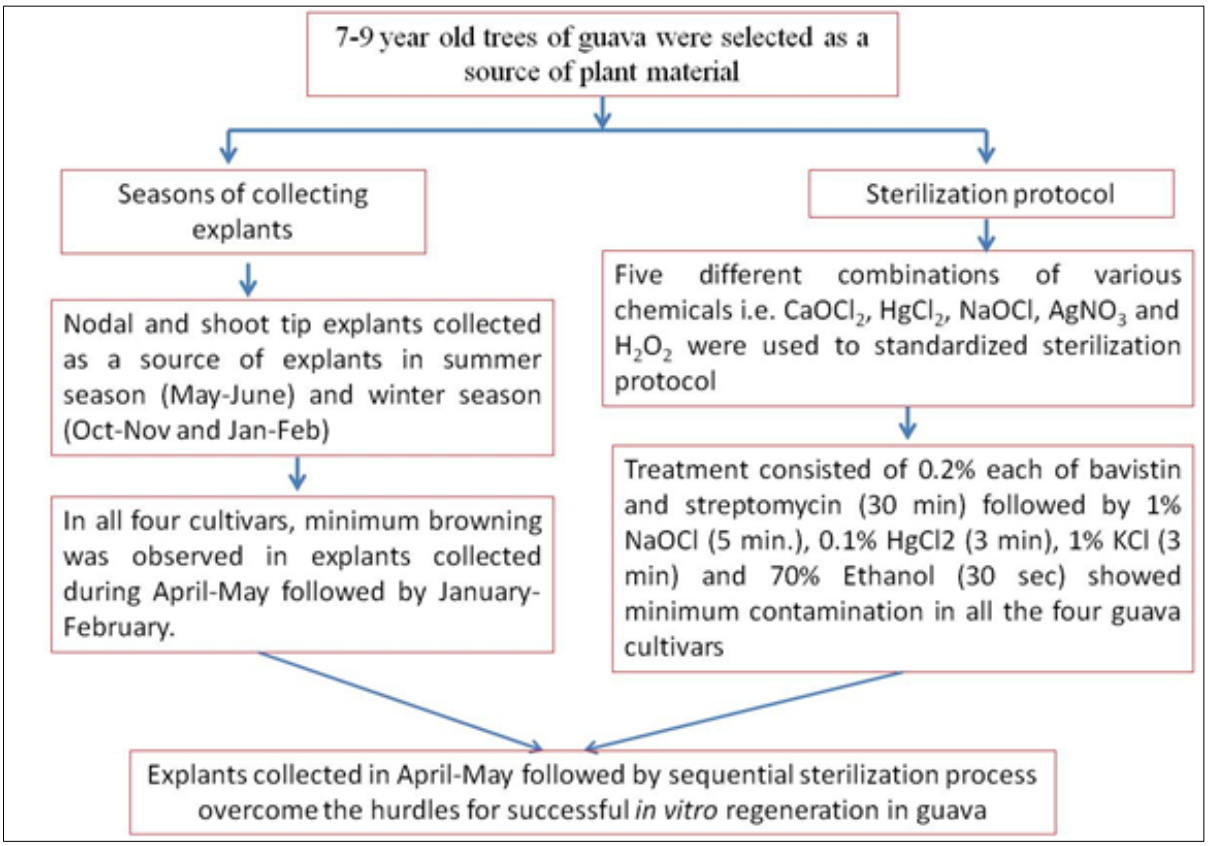


Fig 1: Selected 7 year old mother plant as a source of nodal and shoot tip explants



Shoot tip and nodal explants (2-3 cm) were sterilized using different chemicals *viz*. citric acid, ascorbic acid, NaOCl, $HgCl_2$, KCl, bavistin and streptomycin etc. These explants were collected in 100 ml solution with citric acid (75 mg/l),

ascorbic acid (50 mg/l) and 2-3 drops of tween-20 (polyoxyethylenesorbitan monolaurate) to prevent phenolic secretion while collecting explants. These were further washed thoroughly in running tap water for half an hour to remove dust particles. These explants were surface sterilized with 1% NaOCl for 5 min and again washed with 2-3 times with distilled water which followed by treating with solution of 0.2% bavistin (w/v fungicide) and streptocyclin (0.2% w/v) for 30 min and repeated 2-3 times washing with distilled water. Thereafter, explants were carried in laminar air flow where these further treated with 0.1% $HgCl_2$ (w/v) for 3 min. The explants were dipped in autoclaved solution of 1% KCl (w/v) for 5 min for removing any traces of mercuric chloride sticking on surface of explants and again rinsed with sterile distilled water. Finally explants were treated with 70% ethanol (v/v) for 30 seconds, rinsed with sterile distilled water followed by drying on pre-sterilized filter paper. The surface sterilized explants (shoot tip and nodal) of 1-1.5 cm were inoculated on culture medium. In each bottle, 4-5 explants were inoculated. Cultures were then transferred to the culture room maintained at 26±2ºC and a photoperiod cycle of 16 hrs light and 8 hrs dark through fluorescent tube lights (3000-4000 lux). Visual scoring according to intensity of browning of explants was done for the observation of seasonal effect and percent contamination in culture nodal and shoot tip explants. The data collected was analyzed statically using completely randomized block design (OPSTAT, CCS HAU, Hisar) and mean differences were compared at 5 per cent level of significance and compared with the treatments means.

Results and Discussion

A variety of explants have been successfully used for developing a reproducible micropropagation protocol of guava and the most commonly used explants are nodal and shoot tip, arising from newly grown stem (Amin and Jaiswal., 1987; Ali *et al*., 2003; Zamir *et al*., 2004) [1, 2, 20].

Effect of seasons of collecting explants on *in vitro* propagation of guava

The major hindrance in plant tissue culture of woody plants is browning due to phenol exudation. Plant materials for nodal and shoot tip explants were collected in various seasons and the browning due to phenolics was assessed. The explants were collected in dilute solution of antioxidants, citric acid (75 mg/l), ascorbic acid (50 mg/l) and few drops of Tween 20 as wetting agent. A visual scoring according to intensity of browning of explants was done for the observation of seasonal effect. In all four cultivars, minimum browning was observed in explants which were collected during April-May followed by January-February. The explants collected during July-August and October-November showed maximum browning. The explants collected in April-May showed minimum browning in Hisar Safeda and Lucknow-49 (Table 2). Seasonal variation affects *in vitro* growth and development due to hormonal changes in xylem sap of plant (Alvima *et al.*, 1976; Carsells *et al.*, 1982) [3, 6], which determined the content of phenolic compounds, degree of contamination and *in vitro* response of explants. Several other researcher also adduce the reported findings that explants excised in months of April-May were better for healthy culture establishment in guava (Singh *et al*., 2001; Kumar, 2003; Meghwal *et al*., 2010) [17, 10, 12].

Table 1: Seasonal effect on explant browning in four cultivars of Guava

Months of explants collection	Cultivars			
	Hisar Safeda	Hisar Surkha	Lucknow-49	Allahabad Safeda
January-February	++	++	++	++
April-May	+	++	+	++
July-August	++++	+++	+++	++++
October-November	+++	++++	+++	++++

++++ Very High browning; +++ High browning; ++ Moderate browning; + Low browning

Sterilization of explants

Proper sterilization of explants is the pre-requisite of a protocol to be successful. Media contamination due to unwanted guest particularly bacterial, fungal is one of the most important limiting factors for culture initiation using vegetative plant parts (Krishna and Singh, 2007) because this can result in the death of cultures, altered morphogenic potential and reduced the rates of multiplication (George 1993; Rai *et al*., 2010) [15]. Therefore, the used explants (shoot tip and nodal) of guava were surface sterilized by various chemicals. Five different treatments (T1-T5) were used which included use of calcium hypochlorite, mercuric chloride, sodium hypochlorite, silver nitrate and hydrogen peroxide. The effects of different treatments on sterilization of nodal and shoot tip explants of four elite cultivars are listed in Table 8 and 9 respectively. In cultured nodal explants (Table 3), the minimum contamination of 27.04%, 31.56%, 28.83% and 25.44% was observed in T3 treatment in Hisar Safeda, Hisar Surkha, Lucknow-49 and Allahabad Safeda respectively. It consisted of solution of 0.2% each of bavistin and streptomycin (30 min) followed by 1% NaOCl (5 min.), 0.1% $HgCl_2$ (3 min), 1% KCl (3 min) and 70% Ethanol (30 sec). This might be due to synthesis of less quantity of phenolics in the juvenile explants when grown in controlled environmental conditions (Chandra *et al*., 2005b). Similarly in shoot tip explants (Table 4), 25.04%, 31.04% and 23.96% contamination was observed in Hisar Safeda, Hisar Surkha and Lucknow-49 respectively on T3 treatment, however, Allahabad Safeda showed minimum contamination of 32.62% in T1 treatment which included 0.5% bavistin (20 min), further treatment with 4% CaOCl2 (10 min), 0.1% $HgCl_2$ (3 min) and final treatment with 70% Ethanol (30 sec). The percent contamination data in two explants revealed that contamination was lower in shoot tip explants than nodal explants on T3 treatment. The maximum contamination in nodal explants of Hisar Safeda (73.92%), Hisar Surkha (84.62%), Lucknow-49 (77.66%) and Allahabad Safeda (69.32%) was recorded when explants were treated with 4% NaOCl (5 min.) and 1% AgNO3 (10 min.), 0.1% $HgCl_2$ (3 min.) and 70% ethanol (30 sec.) in T5 treatment. Similarly maximum contamination in shoot tip explants in Hisar Safeda (79.47%), Hisar Surkha (92.91%), Lucknow-49 (75.38%) and Allahabad Safeda (62.53%) was recorded in T5 treatment. Similar findings were also reported by Amin and Jaiswal (1987) [1]; Khattak *et al*. (1990) [11]; Sidddiqui and Farrooq (1997); Meghwal *et al*. (2001) [13]; Bisen and Tiwari (2006) [5] and Zamir *et al*. (2007) [19] and Devi *et al*. (2021) [8] who also used these chemicals separately to standardize the sterilization process to minimize the contamination and successful regeneration in guava.

Table 2: Percent contamination in cultured nodal explants under different sterilization treatments in four cultivars of Guava

S. No.	Treatment Details	Percent Contamination (Mean ± SE)*			
		Hisar Safeda	Hisar Surkha	L-49	Allahabad Safeda
1	0.5% Bavistin (20 min) + 4% $CaOCl_2$ (10 min) + 0.1% $HgCl_2$ (3 min) + 70% Ethanol (30 sec.) – Treatment 1	38.14 (38.12±0.40)	36.63 (37.22±0.99)	64.72 (53.54±0.72)	36.97 (37.43±0.63)
2	0.5% Bavistin (20 min) + 10% H_2O_2 (5 min) + 0.1% $HgCl_2$ (3 min) + 70% Ethanol (30 sec) – Treatment 2	46.09 (42.74±0.49)	52.40 (46.36±3.18)	37.84 (37.93±1.24)	45.56 (42.42±2.12)
3	0.2% each of Bavistin and Streptomycin (30 min) + 1% NaOCl (5 min) + 0.1% $HgCl_2$ (3 min) + 1% KCl (3 min) + 70% Ethanol (30 sec) – Treatment 3	27.04 (31.24± 2.00)	31.56 (34.10±2.12)	28.83 (32.46±0.46)	25.44 (30.24±1.38)
4	0.2% Bavistin (30 min) + 1% $AgNO_3$ (10 min) + 0.1% $HgCl_2$ (3 min) + 70% ethanol (30 sec) – Treatment 4	65.45 (53.98±0.36)	67.95 (55.53±1.55)	44.16 (41.62±0.95)	58.96 (50.14±0.32)
5	4% NaOCl (5 min) + 1% $AgNO_3$ (10 min) + 0.1% $HgCl_2$ (3 min) + 70% ethanol (30 sec) – Treatment 5	73.92 (59.34±2.01)	84.62 (67.02±2.04)	77.66 (61.82±1.47)	69.32 (56.34±0.22)
C.D at 5%		4.18	6.72	3.30	3.76
SE (m)		1.31	2.10	1.03	1.18

*Values in parenthesis show angular transformation data

Table 3: Percent contamination in cultured shoot tip explants under different sterilization treatments in four cultivars of Guava

S. No.	Treatment Details	Percent Contamination (Mean ± SE)*			
		Hisar Safeda	Hisar Surkha	L-49	Allahabad Safeda
1	0.5% Bavistin (20 min) + 4% $CaOCl_2$ (10 min) + 0.1% $HgCl_2$ (3 min) + 70% Ethanol (30 sec.) – Treatment 1	35.51 (36.55±1.01)	45.79 (42.56±1.38)	64.80 (53.51±0.11)	32.62 (34.67±3.14)
2	0.5% Bavistin (20 min) + 10% H_2O_2 (5 min) + 0.1% $HgCl_2$ (3 min) + 70% Ethanol (30 sec) – Treatment 2	44.10 (41.59±0.85)	65.88 (44.59±9.04)	30.05 (33.20±1.33)	51.85 (46.45±1.06)
3	0.2% each of Bavistin and Streptomycin (30 min) + 1% NaOCl (5 min) + 0.1% $HgCl_2$ (3 min) + 1% KCl (3 min) + 70% Ethanol (30 sec) – Treatment 3	25.04 (29.93±2.12)	31.04 (33.48±5.30)	23.96 (29.25±1.43)	50.82 (45.45±0.73)
4	0.2% Bavistin (30 min) + 1% $AgNO_3$ (10 min) + 0.1% $HgCl_2$ (3 min) + 70% ethanol (30 sec) – Treatment 4	54.83 (47.77±2.24)	65.94 (54.43±3.45)	46.63 (43.04±1.06)	56.47 (48.75±3.76)
5	4% NaOCl (5 min) + 1% $AgNO_3$ (10 min) + 0.1% $HgCl_2$ (3 min) + 70% ethanol (30 sec) – Treatment 5	79.47 (63.35±3.61)	92.91 (75.24±3.46)	75.38 (60.32±2.11)	62.53 (52.30±2.90)
CD at 5%		7.04	16.63	4.38	8.34
SE (m)		3.12	5.21	1.37	2.61

*Values in parenthesis show angular transformation data

Conclusion

The explants collected in April-May showed minimum browning in Hisar Safeda and Lucknow-49. In shoot tip and nodal explants of guava, the minimum contamination was noted under treatment consisted of 0.2% each of bavistin and streptomycin (30 min) followed by 1% NaOCl (5 min.), 0.1% $HgCl_2$ (3 min), 1% KCl (3 min) and 70% Ethanol (30 sec). Results obtained from the study concluded that for successful *in vitro* regeneration, especially for difficult and recalcitrant tree species such as guava, is mainly dependent on the source and season of explants collection and the sterilization protocol.

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