Bangladesh J. Bot. **50**(4): 1151-1158, 2021 (December) DOI: https://doi.org/10.3329/bjb.v50i4.57083

SEQUENTIAL STERILIZATION OF BANANA (*MUSA* SPP.) SUCKER TIP REDUCING MICROBIAL CONTAMINATION WITH HIGHEST ESTABLISHMENT PERCENTAGE

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Keywords: Lactic acid, Sodium hypochloride, Sodium chlorite, Mercury chloride

Abstract

Sterilization procedure was standardized for grand naine cultivar of banana using various sterilants in combinations and alone. The observations were recorded regularly with respect to presence of fungal, bacteria as well as percentages of culture establishment. Results indicated that a treatment combination No. 3 [Lactic acid (0.15 %) + Tween-20 (0.1 %) + 0.8 % Commercial bleach (30 Min.) followed by Sodium chlorite (0.3 %) (20 min)] gave the highest percentage of aseptic culture establishment *in vitro* condition. The present study also showed that, single step aseptic inoculation was unable to control endophytic contaminants while sequential treatments were good enough to reduce microbial load as well as increase culture survival.

Introduction

A belonging to Musaceae is the fourth most important crop with a global production of more than 100 million tons per year. Traditionally, farmers used to grow banana plants through suckers, which are restricted due to 5-15 suckers per plant during entire life cycle. As a consequence, they are failed to get better revenue due to yield variation, virus incidence, inherent pathogens and other somaclonal variations (Ngomuo *et al.* 2014). Moreover, the plants do not produce seeds for planting due to triploid nature. Thus, only vegetative or asexual propagation methods can be used. The micropropagation has advantage such as uniform planting material with disease free in nature than conventional suckers grown plants.

One of the most versatile steps of aseptic inoculation is to optimize decontamination treatment for different plant species due to their varied cell wall compositions. The sensitivity of tissue towards different sterilants, time of contact and concentration affects the success of decontamination treatments (Goswami and Handique 2013). These potential biotic factors compete for the available resources during tissue culture and growth of these microbes will lead to signal transduction in plants tissue. The plant tissue produces certain metabolites in response to these microbes. As a result, the explant gets necrosis and culture mortality. These contaminants are not seen all the time during initiation stage; some contaminant appears after several sub culture cycle. Furthermore, sequential sterilization gave better response than single sterilization step.

Scientists around the world were trying to eliminate contamination using various chemical treatments; however, on an average 10-15% plant losses were observed during micropropagation after every subculture cycle (Goswami and Handique 2013). There are various sterilants like mercuric chloride, sodium chlorite, sodium hypochlorite, sodium benzoate, calcium hypochlorite, ethanol (or isopropyl alcohol), hydrogen peroxide, lactic acid, silver nitrate (Oyebanji *et al.* 2009) routinely used in the micropropagation laboratory. The laundry bleach contains sodium hypochlorite as principle compound and having strong bactericidal activity even at trace concentration. During the preparation of sterilants in water, it dissociates into highly active and

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toxic HoCl salt. Calcium hypochlorite is mild in its effect and alternative to sodium hypochlorite for decontaminating explants (Oyebanji *et al.* 2009); Similarly, mercury chloride is the most common, widely used and extensively reported in various literatures, frequently utilized to alleviate microbial load in tissue culture (Mekonnen *et al.* 2013). Lactic acid can penetrate specifically cell membrane of Gram negative microbes with ease. In addition to that combination of lactic acid with other surfactant could be used for various applications in tissue culture to alleviate even Gram positives microbes also (Boomsma *et al.* 2015). Ethanol could be potent sterilizer with phytotoxic at prolonged exposure. Above all factors requires lot of efforts, time, money and material which if not properly handled can lead to complete wipe of the cultures present in the laboratory. Every sterilant has different effect on various tissues and single chemical cannot remove all microbes. Therefore, present study aimed at analyzing combination of various decontaminating chemicals with possible combination and time of exposure during aseptic inoculation of banana sucker.

Materials and Methods

The study was carried out at Department of Plant Molecular Biology and Biotechnology, Navsari Agricultural University, Navsari, Gujarat, India under controlled laboratory condition for two consecutive years during May-June. Large numbers of suckers can be obtained easily during this period every year and seasoning parameters favours culture initiation per explant.

Sword suckers of medium size were collected from farmer's field and washed under tap water. Detergent was also used to remove dirt and other traces of rhyzospheers followed by several washes with sterile distilled water. The roots and other tissues were trimmed to remove additional leaf sheaths. The shoot tips of about 4-5 cm in width and 6-7 cm in length with 3-4 leaf bases were selected for further treatments of decontaminants. The full strength MS basal salts medium supplemented with 6-Benzylaminopurine (BAP) (3 mg/l), sucrose (3%) and agar (0.8%) was prepared and pH of the medium was kept at 5.8. The inoculated suckers were incubated at 25-27^{0}C for 16 hrs light and 8 hrs dark periods. The experiment was repeated two times for two consecutive years to reduce error.

The off white tissue blocks containing one to two leaf base were taken into tissue culture bottle and surface sterilized (Table 1). The experiment was carefully designed to expose explant with water minimally after excision in order to avoid contamination and rotting. Contamination was evaluated 10 days after inoculation and the effects of the sterilizing agents were identified. Treatments No. 3 and 10 contained two stage procedure while rest all followed single sterilization step.

The bacteria and fungi are the main contaminating agents during initiation of micropropagation experiments. Thus, fungal and bacterial contamination percentages of total inoculated bottles along with establishment percentage was also noticed for two consecutive years and their means were subjected to analysis of variance (ANOVA) for testing the significance of the differences between treatments using SAS statistical software package. The 10 plants per replication/per treatment were taken for the analysis. Tukey's Studentized Range (HSD) Test was used for the pair wise comparison of means as sample sizes and treatment were unequal. Treatment means were separated using the Least Significant Difference at $p < 0.05$. The bar diagramme and Tukey's grouping were also computed for interpreting the outcome of the treatments effects and their difference.

The experiment was conducted for two consecutive years in order to finalize treatment with maximum culture establishment and lower contamination percentages. The suckers are generally collected in monsoon season, as a result high microbial load present within them. Thus single and

sequential sterilization steps were used to get rid of endophytes (Gong and Guo 2009). In the present investigation, various chemicals were used to optimize surface sterilization procedure for banana grand naine suckers. The mercury chloride, sodium hypochlorite, sodium chlorite, lactic acid and sodium benzoate are used either alone or in combination at different time intervals.

Table 1. List of the treatments used for decontamination of suckers of banana grand naine.

T1	NaOCl (0.8 %) + Tween-20 (0.1 %) (5 min.)
T2	Sodium benzoate (0.5%) + Lactic acid (0.15 %) (10 min.)
T3	Lactic acid (0.15 %) + Tween-20 (0.1 %) + Commercial bleach (0.8 %) (30 min.) Followed by Sodium chlorite (0.3 %) (30 min.)
T4	NaOCl (0.8 %) + Tween-20 (0.1 %) (10 min.)
T5	Lactic acid (0.15 %) + (NaClO) (0.03%) (30 min.)
T6	Mercuric chloride (2.0 %) NaOCl (2.0 %) + Tween-20 (0.1 %) (10 min.)
T7	Mercuric chloride (0.1 %) + Tween-20 (0.1 %) (5 min.)
T8	Mercuric chloride (0.1 %) + Tween-20 (0.1 %) (10 min.)
T9	Sodium hypochlorite (NaOCl) (0.8 %) + Tween-20 (0.1 %) (10 min.)
T10	Sodium hypochlorite (NaOCl) (0.8 %) + Tween-20 (0.1 %) (20 min.)
T11	Sodium benzoate (0.5%) + Lactic acid (0.15 %) (5 min.)
T12	Sodium benzoate (0.5%) + Lactic acid (0.15 %) (10 min.)
T13	Lactic acid (0.15 %) + Tween-20 (0.1 %) + 0.8 % Commercial bleach (30 min.) followed by Sodium chlorite (0.3 %) (10 min.)
T14	Lactic acid (0.15 %) + Tween-20 (0.1 %) + 0.8 % Commercial bleach (30 min.) followed by Sodium chlorite (0.3 %) (20 min)

Results and Discussion

The optimization of chemical concentration and time of exposure was very important factors as explants must be alive and free from any contaminating material. Initial reports indicated the use of sterilants mentioned in the Table 1. The efficiency and efficacy of disinfection of explant is the most important criteria for any micropropagation technique. In the initial treatment, banana suckers were decontaminated with T3 treatment and gave highest culture establishment (79.68% ± 2.30) with average fungal contamination of 37.5 ± 0% and bacterial contamination 22.5 ± 0 (Table 2). In T3 two step procedures were used and complete removal of contaminants was ensured, while rest others reported single step protocol. The results are very much in conformity with other previous studies on various crops. Likewise, *In vitro* sterilization techniques were optimized for local cultivar as well as grand naine using antibiotics and different sterilants. Two step sterilization procedures used in the preset study as T3, T13 and T14 for different time period. In this experiment, first large size suckers was trimmed and sterilized, followed by removal of one to two leaf base and finally decontamination steps performed in laminar air flow cabinet by some other chemical agents (Madhulatha *et al.* 2004). On the contrary single treatment of NaOCl or sodium benzoate did not have significant effect on culture establishment as well as controlling contamination. Moreover, increasing time of exposure from 5 to 10 min of NaOCl reduces the fungal as well bacterial contamination percentages; as a result, the culture establishment was found to be higher than 5 min incubation (Colgecen *et al.* 2011, Morla *et al.* 2011). Sodium hypo chloride consists of single chlorine atom and its cytotoxic effect is due to production of oxygen from decomposed salt, responsible for bactericidal activity.

Table 2. The effect of different sterilants at different concentration and time intervals on suckers for two succesive years.

Sr. No.	Treatments	Fungal contamination	Bacterial contamination	Culture Establishment
			(Mean % ± S.Em.)	
T1	NaOCl (0.8 %) + Tween-20 (0.1 %) (5 Min.)	57.5±0.57^{c}	37.5±1.15^{ab}	56.68±0.76^{c}
T2	Sodium benzoate (0.5%) + Lactic acid (0.15 %) (10 Min.)	47.5±0.43^{d}	27.5±2.22^{cd}	66.68±0.75^{b}
T3	Lactic acid (0.15 %) + Tween-20 (0.1 %) + Commercial bleach (0.8 %) (30 Min.) followed by Sodium chlorite (0.3 %) (30 Min.)	37.5±0^{e}	22.5±0^{d}	79.68±2.30^{a}
T4	NaOCl (0.8 %) + Tween-20 (0.1 %) (10 Min.)	52.33±0.72^{cd}	32.5±0.28^{bc}	68.78±0.64^{b}
T5	Lactic acid (0.15 %) + (NaClO) (0.03%) (30 Min.)	82.5±3.37^{a}	42.83±1.16^{a}	45.82±0.003^{d}
T6	$HgCl_2$ (2.0 %) NaOCl (2.0 %) + Tween-20 (0.1 %) (10 Min.)	67.5±1.24^{b}	37.5±1.44^{ab}	33.30±1.17^{e}
Minimum Significant Difference		8.13	5.73	5.58
T7	Mercuric chloride (0.1 %) + Tween-20 (0.1 %) (5 Min.)	66.30±0.67^{b}	54.05±0.58^{bc}	50±1.15^{de}
T8	Mercuric chloride (0.1 %) + Tween-20 (0.1 %) (10 Min.)	54.17±0.67^{c}	55.10±2.03^{bc}	45.81±0.46^{fe}
T9	Sodium hypochlorite (NaOCl) (0.8 %) + Tween-20 (0.1 %) (10 Min.)	50±0.57^{cd}	41.63±0.34^{d}	58.31±0.40^{bc}
T10	Sodium hypochlorite (NaOCl) (0.8 %) + Tween-20 (0.1 %) (20 Min.)	45.83±0.47^{d}	50.33±1.45^{cd}	54.17±2.21^{cd}
T11	Sodium benzoate (0.5%) + Lactic acid (0.15 %) (5 Min.)	79.17±2.25^{a}	76.14±4.74^{a}	41.67±0.38^{gf}
T12	Sodium benzoate (0.5%) + Lactic acid (0.15 %) (10 Min.)	70.29±1.07^{b}	62.5±2.59^{b}	37.5±0^{g}
T13	Lactic acid (0.15 %) + Tween-20 (0.1 %) + 0.8 % Commercial bleach (30 Min.) followed by Sodium chlorite (0.3 %) (10 Min.)	33.33±1.34^{e}	29.17±1.69^{e}	62.5±1.44^{ab}
T14	Lactic acid (0.15 %) + Tween-20 (0.1 %) + 0.8 % Commercial bleach (30 Min.) followed by Sodium chlorite (0.3 %) (20 min)	29.17±0.67^{e}	25.03±1.05^{e}	66.67±0.83^{a}
Minimum Significant Difference		4.81	9.33	4.61

Data were the means from the three replicated experiments each with 15 suckers. Means followed by the same letters are not statistically significant (p = 0.05) obtained by Tukey's Studentized Range (HSD) test. SE = standard error.

Sodium hypochlorite is more effective decontaminating agents, which replaced low concentration of mercury chloride for surface sterilization of banana suckers (Molla *et al.* 2004, Titov *et al.* 2004). Whereas, Zemene and Worku 2018 proved that, $HgCl_2$ was better sterilant than NaOCl in reducing contamination rate in micro-propagation of *Green pepper* (*Capsicum annum* L.). Treatment T4, gave 68.78 ± 0.64 culture establishment percentage followed by T2 (66.68 ± 0.75), on the other hand the fungal as well as bacterial contamination percentages were almost similar in both T4 and T2. Surprisingly, T1 had lesser time of exposure than T4, even though, the

T1 showed less culture establishment (56.68 ± 0.76) with higher fungal (57.5 ± 0.57) and bacterial (37.5 ± 1.15) contamination percentages. Treatment 5 had only lactic acid and sodium chlorite as its active ingredient. T5 was unable to control microbial contamination (82.5 ± 3.37 for fungal and 42.83 ± 1.16 for bacteria) as well as shown to be inhibitory to the suckers (45.82 ± 0.003). Likewise, T6 also found to be harsh (33.30 ± 1.17) for the sucker and ineffective in removal of microbial contamination. The culture establishment along with low microbial load was the main criteria for optimization; therefore, the present study further modified for the next trial in next season. From previous data, T1 to T4 were used as ideal treatments for further refining for better culture establishment with minimum microbial load by increasing or decreasing the exposure timing. Mercury chloride at the concentration of 2% was detrimental for plants as in T6 and also contains sodium hypochlorite; thus in T7, the concentration was reduced to 0.1%. Increasing exposure time along with other chemicals in combination significantly reduced the contamination; however, it adversely damage the plant tissues, and hence the culture establishment.

There was significant increase in the culture establishment percentages (50 ± 1.15) with almost similar fungal contamination percentages, while increasing bacterial contamination (54.05 ± 0.58) observed in T7. Most importantly, mercury chloride may be fungicidal as at higher exposure time of 10 min (T8). It showed maximum effects against fungus but lower establishment percentage than T7. However, Exposure times of 5 and 10 min of mercury chloride had no significant affect on bacterial load. The work on the previous season laid foundation for further optimization with respect to time of exposure. Therefore, T4 was repeated as T9 along with modified T10 (Sodium hypochlorite (NaOCl) (0.8 %) + Tween-20 (0.1 %) (20 Min.)). Chlorite radical is released as bichloride of mercury from $HgCl_2$, which is extremely cytotoxic and lethal because of very effective role of two chloride atoms. Moreover, mercury has tendency to bind with proteins to kill the pathogens. Increasing time of interaction of sodium hypochlorite with explant did not significantly affect their establishment percentages (T9: 58.31 ± 0.40, T10: 54.17 ± 2.21) while, bacterial contamination percentages increases over time with reduction in fungal contamination percentages marginally in both the treatments. In the T6 treatment the culture establishment was found to be only 33.30 % and microbial contamination was found to be higher. This is due to the artifacts and growth of microbes on the dead tissues. Synergetic effect of chlorine concentration along with long exposure time had toxic effect of culture as well as microbes.

Similarly, 90% aseptic culture establishment was observed using combination of Sodium hypochlorite (1.0%) for 15 min. followed and $HgCl_2$ (0.1%) for 7 min.; while, none of the chemicals alone for different time did not showed highest aseptic establishment (Goswami and Handique, 2013). Basically, T2 was selected for further optimization as it showed better establishment percentages. When performed for second consecutive year, the establishment percentages dropped down to 37.5 ± 0; however, reduction in time for 5 min. marginally increases its survival. Nonetheless, fungal and bacterial contamination was increased to 79.17 ± 2.25 and 76.14 ± 4.74 respectively (T11). Exposure of mercury chloride for longer period of time leads to the browning of explants and death (Sen *et al.* 2013). In the present investigation, sodium benzoate, lactic acid along with sodium hypo chloride did not show any good response (T2, T5 and T11). Yadav *et al.* 2017 studied aseptic inoculation and culture establishment of banana cv. Grand naine with three different surface sterilization agents i.e. mercuric chloride, bavistin and ethanol. They obtained minimum contamination with higher survival percentages at 0.1 % $HgCl_2$ for different time intervals in banana cv. Grand Naine. The 0.1% HgCl2 in combination with 70% ethanol gave significant results during sterilization of explants. The 0.1% Mercury chloride for 6 min showed minimum contamination and highest culture establishment (Onuoha *et al.* 2011). The present results are practically the same as to previous reports by Onuoha *et al.* (2011), Jaisy and

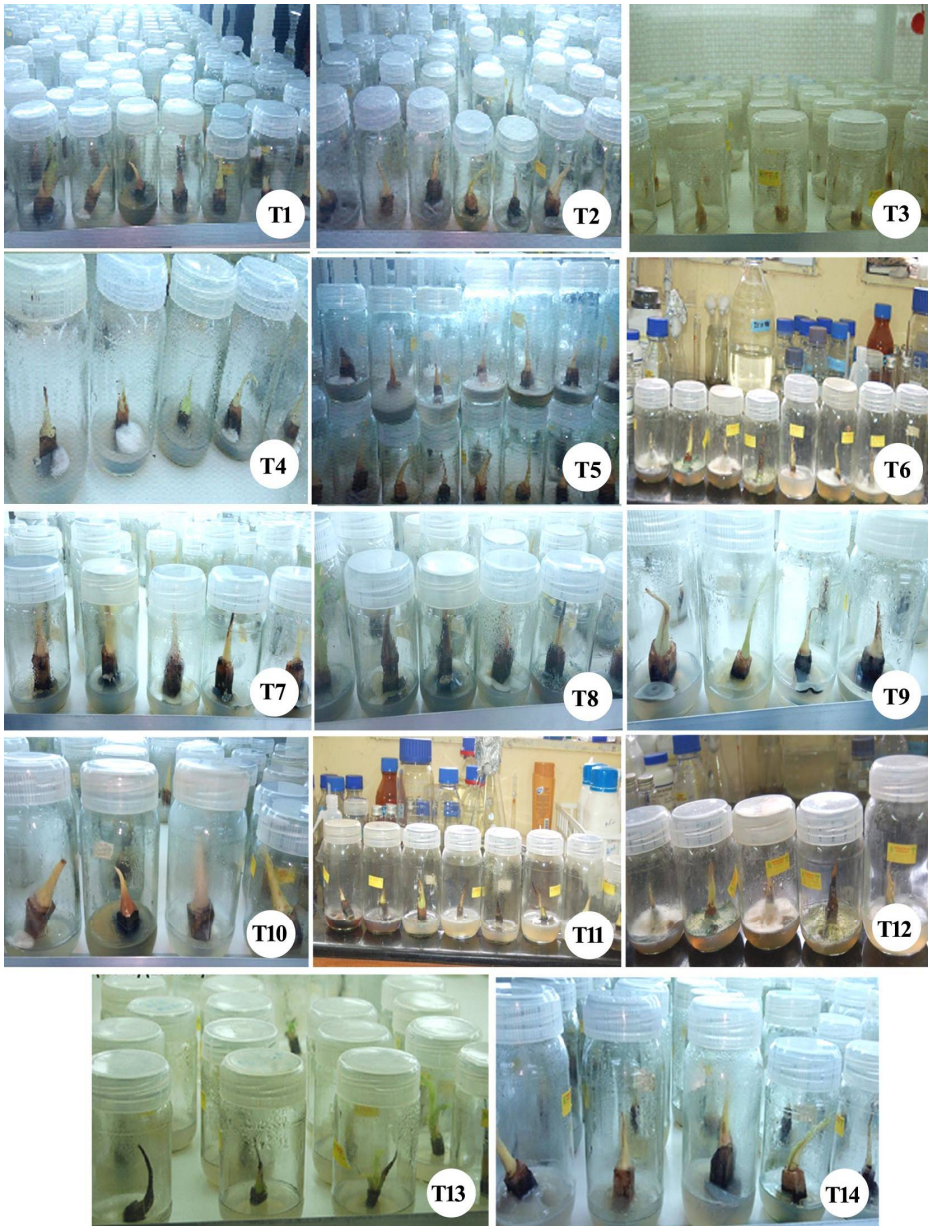

Fig. 1. Effect of different treatments of sterilants on fungal load, bacterial load as well as culture establishment percentage. T1. NaOCl (8%) + Tween-2 (0.1%) (5 min), T2. Sodium benzoate (0.5%) + Lactic acid (0.15%) (10 min), T3. Lactic acid (0.15%) + Tween-20 (0.1%) + Commercial bleach (0.8%) (30 min) followed by sodium chlorite (0.3%) (30 min), T4. NaOCl (0.8%) + Tween-20 (0.1%) (10 min), T5. Lactic acid (0.1`5%) + (NaClO) (0.03%) (30 min), T6. $HgCl_2$ (2.0%) NaOCl (2.0%) + Tween-20 (0.1%) (10 min), T7. Mercuric chloride (0.1 %) + Tween-20 (0.1 %) (5 Min), T8. Mercuric chloride (0.1 %) + Tween-20 (0.1 %) (10 Min), T9. Sodium hypochlorite (NaOCl) (0.8 %) + Tween-20 (0.1 %) (10 Min) T10. Sodium hypochlorite (NaOCl) (0.8 %) + Tween-20 (0.1 %) (20 Min), T11. Sodium benzoate (0.5%) + Lactic acid (0.15 %) (5 Min), T12. Sodium benzoate (0.5%) + Lactic acid (0.15 %) (10 Min), T13. Lactic acid (0.15%) + Tween-20 (0.1%) + 0.8% Commercial bleach (30 min.) followed by Sodium chlorite (0.3% (10 min.) (0.5%) + Lactic acid (0.15 %) (5 Min), T14. Lactic acid (0.15 %) + Tween-20 (0.1 %) + 0.8 % Commercial bleach (30 Min.) followed by Sodium chlorite (0.3 %) (20 min).

Ghai (2011), Sen *et al.* (2013) and Ahmed *et al.* (2014) had used ethanol along with $HgCl_2$ for decontamination purpose.

In the present study, increase of duration of sterilization has negative effect on culture establishment due to sensitivity of inner leaf base towards sterilants. There were two successive sterilization steps performed and found to be more effective against endophytes as well as surface contaminant. Among all the treatment, T3 gave minimum contamination with highest culture establishment percentage than others. The same treatment composition and steps were further tested at different time interval. All the methods mentioned in the Table 1 had single sterilization steps, while T3, T13 and T14 had two sequential sterilization steps. The most surprising results were obtained while time of exposure was decreasing. Bacterial contamination increases with T14 (20 min.) and T13 (10 min.) respectively than T3 (30 min.). At the same time, the percentage of establishment was also reduced from 79.68% ± 2.30 (T3) to 62.5 ± 1.44 (T13). The time of exposure of 30 min. at final sterilization steps significantly affects culture establishment. Therefore, T3 proved to be the best among all the treatment studied for the decontamination with higher culture establishment. Data indicated that there was a significant variation and response at 0.05 *P* level in contamination percentage and culture establishment among the different treatments.

There were altogether T1-T14 treatments used in this experiment in order to optimise decontamination treatment. Initially it was started with 6 treatments and in the subsequent year's number increased to 14 with some modified treatments. The treatment number T3 gave good commercially viable results with lowest fungal contamination and bacterial contamination percentage compared to others used in this experiment. Hence this decontamination treatment is considered as best to control initial deep seated endophytic contamination for culture initiation in the micro propagation of banana cv. Grand Naine from sucker tips. Results suggested that use of Lactic acid (0.15 %) + Tween-20 (0.1 %) + Commercial bleach (0.8 %) (30 Minutes) followed by Sodium chlorite (0.3 %) (30 Minutes) might be used as decontamination methods for commercial purpose.

Acknowledgements

The authors gratefully acknowledge the Chairman, Department of Plant Molecular Biology and Biotechnology, ACHF, NAU, Navsari, Gujarat India for providing necessary funds and laboratory facilities to carry out the work.

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(Manuscript received on 17 May, 2021; revised on 25 September, 2021)