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Supplementation of Urea as the Nitrate Source of MS Medium and High-frequency Regeneration of Plantlets of *Musa paradisiaca* cv. Malnad Rasbale

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ABSTRACT

Background: A consistent practice for mass multiplication of banana cv. Malnad Rasbale has been accomplished, which is an endemic cultivar of Western Ghats of Karnataka, India.

Methods: Decrease in microbial contagion have been managed by dealing with outward sterilization agents ethanol, sodium hypochlorite, mercuric chloride solution, antibiotic solutions containing cefotaxime and gentamicin. MS media augmented with 350 mg/l urea as the nitrogen source by substituting KNO_3 and NH_4NO_3 which is commonly used in terroristic activities. Organogenic media was supplemented with a range of 2-6 mg/l BAP and 0.3-2.7 mg/l TDZ.

Result: The addition of urea substituting KNO_3 and NH_4NO_3 gave the eminent grades in overall initiation, shoot multiplication and development and also in the rooting phase. Supplementation for shoot development with BAP (4 mg/l), TDZ (1.8 mg/l) and with 350 mg/l urea showed enthusiastic progress in shoot elongation with specific concentrations. Plants established under *in vitro* circumstances were reassigned to the greenhouse and after the progress, they were promoted to field conditions.

Key words: *Fusarium oxysporum,* Malnad rasabale, Micropropagation, Nitrogen source, Urea.

INTRODUCTION

Many taxa of bananas are remained to be described due to various factors like existence in dense evergreen forests, civil unrest in the region, *etc.*, within the center of diversification in different parts of India. (Sabu *et al.* 2013; Joe *et al.* 2013). Rasathali (AAB) is one of the most popular banana cultivars in South India. In Karnataka State, India Nanjanagud Rasabale and Malnad Rasabale are the popular cultivars recognized for their special flavor and taste. Nanjanagud Rasabale is recognized as a geographical indicator fruit (Kishor *et al.* 2017) and many investigators standardized the protocol for the micropropagation of plantlets (Bohra *et al.* 2013). The cultivar Malnad Rasabale (silk AAB group) is endemic to the evergreen region of central Western Ghats of Karnataka, India which is popularly known as the Malnad region. A Survey of the literature indicated that standardization of micropropagation protocol has not been investigated so far on the cultivar of banana. This cultivar is highly susceptible to Panama disease caused by the pathogenic fungus *Fusarium oxysporum* f.sp. cubense (FOC) and the farmers are struggling to get disease-free healthy planting materials.

Standardization of efficient protocol for the *in vitro* micropropagation is essential to get efficient *in vivo* developed replicates. To establish the *in vitro* raised plants in field conditions here proper development of rooting and acclimatizing of *in vitro* shoot regeneration and micropropagation is required (David *et al.* 2018). Many investigators established efficient and commercially applicable protocols using Murasshige and Skoog's (1961) nutrient formulation for the mass production of banana cultivars grown in different agroclimatic regions of India (Ngomuo *et al.* 2014).

Nitrates are the main macronutrients essential for the growth of plants. In the absence of these plants, growth is impaired *and* metabolic activities are hindered. The biomolecules namely, amino acids, proteins, coenzymes *and* nucleic acids are the main component of plant growth (Silva Junior *et al.*2013) but for the biosynthesis of these molecules, plants required nitrates (Marschner, 2012). As banana plants grow in different habitats, they will be exposed to different kinds of organic, minerals *and* nitrogen forms. With nitrate being abundant in the soil it appears in nitrate ($N\text{-}NO_3$) or ammonia ($N\text{-}NH_4$ þ) forms (Shan *et al.* 2012). In most of the nutrient formulations (MS, LS, Gambergs B, *etc.*,) ammonium nitrate and potassium nitrate are supplemented as the main macronutrient source. Due to terroristic, mining *and* illegal activities supply and use of ammonium nitrate are forbidden in most countries (Pasman *et al.* 2020). This

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affects the tissue culture research and industries and supplementation of nitrogen sources is very essential for the growth and multiplication of plantlets. Therefore this investigation aims to develop an efficient protocol for the *in vitro* mass multiplication of the endemic banana cv. Malnad Rasabale and comparatively analyze the effect of Urea with the nitrate sources of MS media on the rate of regeneration of propagules in *in vitro* conditions.

MATERIALS AND METHODS

Collection of plant material

Healthy 4-5 weeks well-established sword suckers of *Musa paradisica* cv. Malnad Rasbale with unvarying sizes were identified first from the exclusive banana plants which had a high elevation of fruits and were correspondingly free from disease-free mother plants. These healthy clones were detached from the pseudostem fragment of the mother plant which was 6-7 inches beyond the soil surface level and collected from banana farmyards of Malnad regions of Karnataka, India.

Surface sterilization

The first step of sterilization of sword suckers was carried out by thoroughly washing with running tap water, then they were soaked overnight in a solution of 0.3-0.4% bavistin (fungicide) in a container. The outer sheath of the trimmed sword sucker was again trimmed to 1-2 inches of length and breadth and it was dipped in 1-2 drops of tween 20 (polyoxyethylene sorbitan monolaurate) solution mixed with distilled water for 1 hour and again washed thoroughly to remove the tween 20 residuals. By using these trimmed clones containing 3-4 leaf sheths measuring 2-3 cms were taken out for the further process.

Trimmed banana clones were bought to laminar airflow. Then they were surface-sterilized for 2-3 minutes in 70% ethanol and distilled water for 5 minutes. 0.2% Mercuric chloride solution was used again to surface sterilize with a time duration of 4-5 minutes and 2-3 times repeatedly rinsed with sterile distilled water to remove the $HgCl_2$ residues for 5 minutes. Excluding another leaf sheath, only one sheath was removed *and* the rhizosphere part was slightly trimmed to remove the outer layer and was immersed in 2% sodium hypochlorite for 10-15 minutes, to remove the sodium hypochlorite residues banana clones were rinsed thoroughly with distilled water for 5 minutes twice. Antibiotic solutions containing cefotaxime 0.1-0.2% and Gentamicin 0.05% were prepared. The suckers thoroughly washed with distilled water were again further trimmed and immersed in antibiotic solution. Ascorbic acid with a concentration of 100mg/l was used for the treatment suckers after the treatment with antibiotics to avoid phenolic exudation. (Kumar R.S. *and* Krishna. 2015).

Micro-propagation and Acclimatization of explant

Surface sterilized, antibiotic-treated *and* ascorbic acid-treated suckers were inoculated on MS media (Murashige *and* Skoog 1962), augmented with 0.8% agar and 30 g/l sucrose. Growth regulators were added at different concentrations with the combination of BAP at 2-6 mg/l and TDZ at 0.3-2.70 mg/l. the pH of the media was adjusted between 5.6-5.8 and the media was autoclaved. On the other hand addition of urea replacing potassium nitrate and ammonium nitrate as macronutrients were also tested.

Control in light and temperature in the incubation room for culture growth conditions is preferred MS media supplemented with growth regulators was used for culture incubation. Cultures that were inoculated were incubated in the growth chamber at 16 hours of photoperiod and 28±2°C, illuminated by fluorescent tube light (40 watts). After the incubation explants with contamination-free and healthy conditions will be transferred for the multiplication stage.

Mass multiplication, shoot *and* root development of propagules

Fine grown initiation cultures were marked vertically with the help of autoclaved scalpel and were transferred MS media containing 2-6 mg/l BAP and TDZ 0.3-2.70 mg/l and also the addition of urea as a macronutrient, for the development of multiple shoot buds. The incubation period of inoculated cultures was around 3-4 weeks. Here multiplication of inoculated shoots was carried out up to the 5^{th} stage with media supplemented with the growth regulators of the same concentration.

From the first stage of a subculture here blackening of explants will be seen. To avoid this the outer layers of the explant will be removed with the help of a sterilized scalpel and transferred to fresh media frequently after the excess phenolic content is released from the explant. After every subculture cycle here the observation was carried out to check the microbial contamination which appears after 4-5 days of inoculation. The shoot propagules developed on subculture media were transferred to shooting media composed of the above-mentioned growth regulators after the 5^{th} stage of multiplication. After the shooting stage, the regenarants were transferred to rooting media and were incubated to develop the roots for further investigation. MS basal media containing 0.25-2.50 mg/l NAA, 0.25-2.50 mg/l IBA was used for rooting media along with 0.2% activated charcoal to initiate the root formation. The observation was achieved on the root development from the first day of inoculation to the 4^{th} week of time duration, data were recorded.

Hardening of the regenerants in polyhouse conditions

Fine-grown plantlets in rooting conditions were selected and carefully taken out and washed smoothly in water to remove the gelling contents stuck to roots. The regenerants were treated with 0.2% Bavistin for up to 5 min and were further transferred to coco peat which are the fine powder of the outer sheath of coconut. Coco peat containing plants were watered and were covered using poly sheets to create pressure inside.

Plantlets inside the cockpits were transferred to polybags containing soil, sand *and* cow dung manure with a ratio of 1:1:2 ratio and were left to grow for up to 45 days under natural light with proper watering. Fine-grown plants were transferred to field conditions. All the data regarding the development of suckers and multiplication of suckers by the effect of auxins and cytokinins were analyzed by using ez ANOVA software.

RESULTS AND DISCUSSION

Media fortified with the addition of urea replacing Nitrates as a macronutrient, banana clone was inoculated (Fig A). Growth of the shoot was observed after 15-17 days under aseptic conditions like globular structures (Fig B). In Malnad Rasbale with the addition of auxin IAA 0.2 mg/ltr in combination with BAP, shoot proliferation was observed followed by variation in urea supplement replacing NH_4NO_3, KNO_3 (Fig C). Concerning 18-21st, the day of observation of subcultured explant uncomparable growth within the multiplication of shoots was observed on the same type of media. The improved numeral of sprout was perceived up to 350 mg/l urea elsewhere this attentiveness (Fig D). Both the triplicates were compared with developmental patterns day by day (Fig E). Media fortified with urea showed the best shoot and root proliferation (Fig F). Shoots after separation from the multiples were transferred to the root initiating a media concentration of 0.2% activated charcoal (Fig G). Fine-grown plants were selected and transferred to the greenhouse for primary hardening in coco peat (Fig H). Polyhouse grew banana plants in coco peats were further transferred for secondary hardening in the greenhouse for one month by the accurate supply of water and further transferred to field conditions (Fig I).

Compared to conventional planting of here *in vitro* micropropagation of bananas provides many advantages due to vigorous multiplication rate, biological uniformity, availability of disease-free material all the year *and* vigorous growth in the early stages (Vuylsteke 1989; Daniells *and* Smith 1991).In our recent investigation, standardization of MS media for the micropropagation of endemic cv. Of banana Musa paradisiac cv. Karibale Monthan by using a shoot tip as an explant (Kumar R.S. *and* Krishna V. 2015). For the establishment of microbial contaminant-free culture in banana micropropagation antibiotics such as cefotaxime and gentamicin are used. (Habiba *et al.* 2002).Treatment with 70% ethanol for 2-3 min, 0.2% Mercuric chloride solution for 4-5 minutes , 2% sodium hypochlorite for 10-15, 0.2% Cefotaxime and 0.5% Gentamicin for 15 min helped in establishing a contamination-free and 78% survivance of shoot tip of cv. Malnad Rasbale is shown in Fig 2 and Table 1. Here surface sterilization plays an important role in the shoot tip culture of Malnad Rasbale.

Skipping some sterilization methods in an early stage will result in the decline of the existence of explants that may get contaminated. Growth inhibition and brownish appearance in media occurred after an 8-9th day of inoculation that was because of the release of phenols by the explant as reported by Strosse *et al.* (2004). Therefore

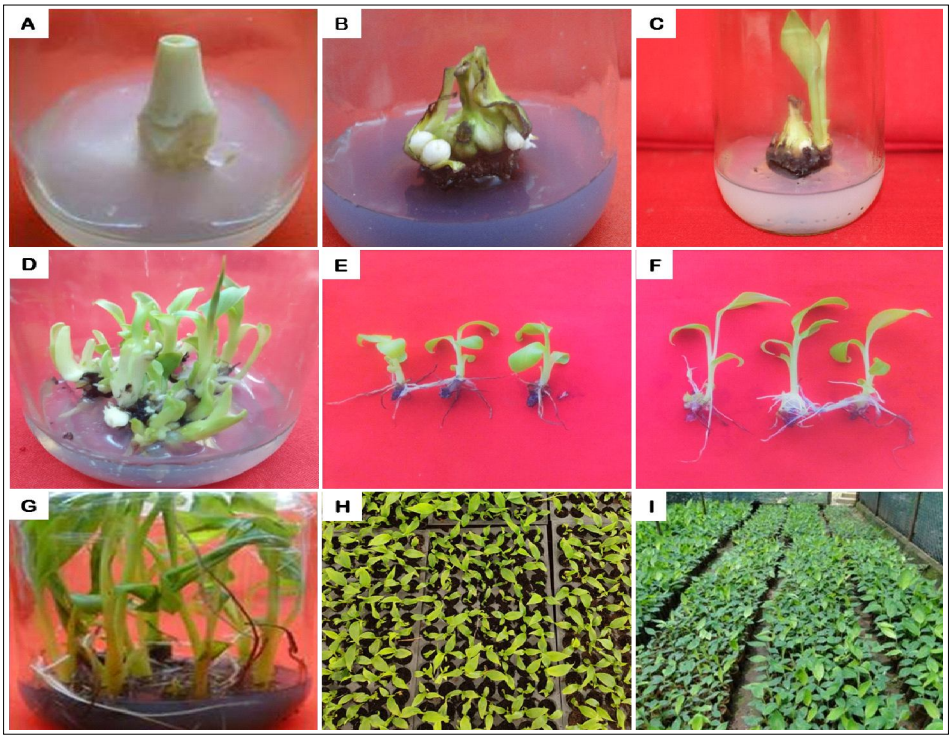

Fig 1: Major steps from *invitro* conditions to greenhouse conditions of the Malnad Rasbale plantlets micropropagation and mass multiplication.

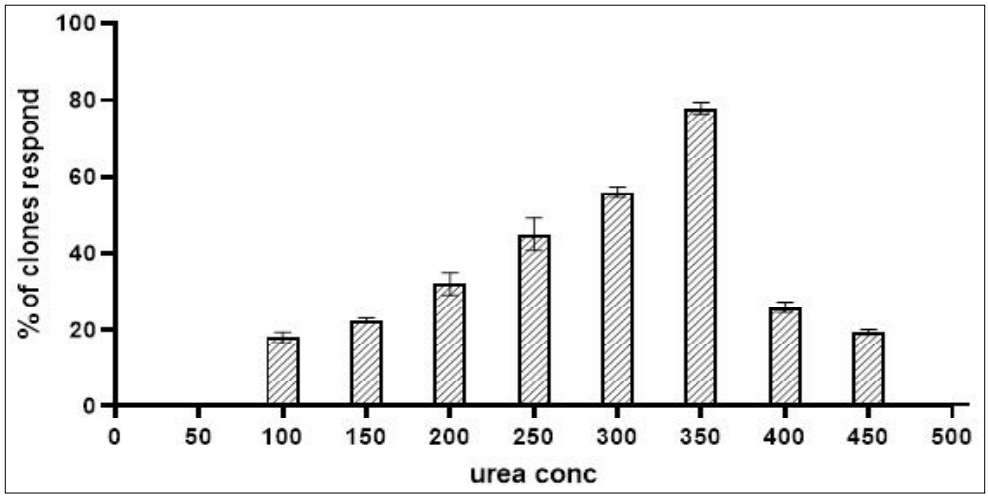

Fig 2: 78% survivance of shoot tip of cv. Malnad Rasbale as shown in Fig A.

Table 1: Effect of urea as nitrate source on shoot induction in primary initiation.

Urea conc. in mg/ltr	% of clones responded
50 mg	0
100 mg	18%
150 mg	22%
200 mg	32%
250 mg	45%
300 mg	58%
350 mg	78%
400 mg	26%
450 mg	18%
500 mg	0%

to overcome the phenolics execution in media explants were treated with ascorbic acid for 10 min at 100 mg/l before inoculation (Kumar R.S. and Krishna 2015) which is an antioxidant that reduces the browning of explants by inhibiting the oxidation of accountable substrates as reported by George in 1996 for many monocotyledonous species. The awareness of standardizing hormonal levels for explants results helps in minimizing many investigations to design the correct hormonal concentrations for shoot development. (Mercier *et al.* 2003). The concentration of hormones plays a major role in the development of tissues in plants, it also varies from plant to plant and cell to cell. (White and Rivin, 2000; Gokani *et al.* 1998). Hence, hormones play a major role in balancing and promoting plant development. (White *et al.* 2000).

At the present investigation slight alteration in MS basal media Urea 50-500 mg/l replacing ammonium nitrate and potassium nitrate gave the best results from initiation of explant stage as mentioned in Table 1. Amino acids, proteins, coenzymes and nucleic acids are the main component for plant growth (Silva Junior *et al.* 2013) but the most required by plants are the nitrates (Marschner 2012). Growth of shoot tip was notified at 4 mg /ltr BAP and 1.80 mg/l TDZ and 350 mg/l urea and was optimized. After the observance of the growth of the shoot tip after 15 days, small globular-shaped propagules appeared on the inoculated explant Fig B. Response by shoot tip stopped while the concentration of urea increased (Table 1).

In the present study, results showed that 4mg/l BAP and 1.80 mg/ltr of TDZ showed the best optimal growth of shoot development in Musa paradisiac cv. Malnad Rasbale. MS media supplemented with cytokinins helped in multiplications of shoots. Well-defined tiny shoots were raised after a time interval of 12-15 days through subculture methods. The rate of multiplication of shoots here was notified after 15 days of the time interval. For the fine development of multiplication of propagules vertically cross half sliced clones were cultured on MS media supplemented with 2-6mg/l BAP and 0.3-2.70 mg/l TDZ and 50-500 mg/l Urea. The outcome of the result was that all the shoots were multiplied in all combinations of cytokinin supplementation and significant difference in the multiplication of shoots were notified in different treatments of growth regulators.

Frequency of 12.23±0.58 multiple shoots in BAP and 14.40±0.70 multiple shoots in TDZ (Fig D) was notified. Here multiplication rate in TDZ was higher than BAP fortified with 350 mg/l urea hence 4mg/l of BAP and 1.50 mg/l TDZ, supplemented with 350 mg/l urea was considered the best combination for maintenance of cultures in good conditions for multiplication rate. Our investigation proved that either TDZ or BAP can be used for the multiplication of shoots with urea but cost-effectiveness BAP can be used. An increase in TDZ combination up to 1.5 mg/l TDZ decreased multiplication potency in the banana cultivars, 'Kibuzi' and 'Bwara' as reported by Arinaitwe *et al.* 2000, but in *Musa paradisica* cv. Malnad Rasbale TDZ supplementation with 1.50 mg/l showed the best results. In many banana genotypes, investigators till today reported that TDZ is one of the effective cytokinins for the development of shoot propagules. (Thomas and Katterman, 1986; Huetteman and Preece, 1993).

Multiplied shoots were transferred to elongation in shooting media supplemented with 2-6 mg/l BAP on the other hand 0.3-1.80 mg/l TDZ with an optimal concentration of urea at 350 mg/l. Elongation of the shoot was recorded highest at 4.5 mg/l BAP (2.90±0.06) (in cm), 1.8 mg/l TDZ (3.39±0.17) (in cm), but the combination of urea with these individual cytokinins showed the best results as followed by 4 mg/l BAP (3.40±0.12) (in cm), 1.8 mg/l TDZ (4.43±0.09) (in cm). Here results concluded that a combination of urea showed the best results in shoot elongation (Table 2) Finely developed shoots of the banana cultivar were transferred to rooting media with the addition of auxins (IBA *and* NAA) for the root development along with activated charcoal with a concentration of 200mg/l. (Kumar and Krishna 2015).

Table 2: Comparison with MS. Media and modified MS media on the shoot development of *Musa paradisica* cv. Malnad Rasbale.

Ms media		Optimized conc of urea at 350/l		Length of shoot developed in cm in BAP and TDZ Conc	
BAP mg/l	TDZ mg/l	BAP mg/l	TDZ mg/l	Ms media	Optimized conc of urea at 350/l
2.0		2.0		1.53±0.15	1.50±0.21
2.5		2.5		1.73±0.22	2.37±0.12
3.0		3.0		2.27±0.12	2.45±0.25
3.5		3.5		2.53±0.15	2.70±0.21
4.0		4.0		2.40±0.15	3.40±0.12
4.5		4.5		2.90±0.06	3.23±0.09
5.0		5.0		1.60±0.18	3.20±0.06
5.5		5.5		1.57±0.20	2.53±0.03
6.0		6.0		1.17±0.13	1.83±0.12
	0.3		0.3	1.40±0.06	1.47±0.24
	0.6		0.6	2.20±0.08	2.37±0.14
	0.9		0.9	2.40±0.18	2.80±0.06
	1.2		1.2	2.73±0.16	3.23±0.09
	1.5		1.5	3.30±0.15	3.73±0.12
	1.8		1.8	3.39±0.17	4.43±0.09
	2.1		2.1	2.50±0.21	3.33±0.15
	2.4		2.4	2.07±0.11	2.67±0.12
	2.7		2.7	1.77±0.09	2.45±0.18

Table 3: Comparison with MS. Media and modified MS media on the root development of *Musa paradisica* cv. Malnad Rasbale.

Ms media		Optimized conc of urea at 350/l		Activated charcoal	Length of root developed in cm in NAA and IBA conc	
NAA mg/l	IBAmg/l	NAA mg/l	IBA mg/l	in mg/l	Ms media	Optimized conc of urea at 350/l
2.0		2.0		200	2.37±0.15	2.87±0.53
2.5		2.5		200	2.53±0.18	3.33±0.61
3.0		3.0		200	2.20±0.11	3.20±0.40
3.5		3.5		200	3.27±0.17	3.47±0.41
4.0		4.0		200	3.50±0.06	3.90±0.75
4.5		4.5		200	4.70±0.21	5.60±0.53
5.0		5.0		200	4.10±0.33	6.50±0.57
5.5		5.5		200	3.40±0.26	4.43±0.44
6.0		6.0		200	3.25±0.16	4.10±0.31
	0.3		0.3	200	2.70±0.23	2.90±0.81
	0.6		0.6	200	3.10±0.26	3.55±0.61
	0.9		0.9	200	3.41±0.23	4.33±0.89
	1.2		1.2	200	3.50±0.35	4.21±0.69
	1.5		1.5	200	4.20±0.36	5.60±0.76
	1.8		1.8	200	4.1±0.29	3.56±0.90
	2.1		2.1	200	3.1±0.36	3.1±0.61
	2.4		2.4	200	2.9±0.61	2.3±0.75
	2.7		2.7	200	2.0±0.31	1.9±0.65

Investigation regarding supplementation of auxins proved that MS media fortified with 4.5 mg/ltr NAA and 1.5 mg/l IBA with separate concentrations develop the roots at the rate of 4.70±0.21 in cm and 4.20±0.36 in cm respectively (Fig E and F). But supplementation of urea showed the best results when compared to MS media (Table 3). Development of roots was observed in urea concentration of 350 mg/l, NAA at 6.50±0.57 (in cm) and on the other hand 5.60±0.76 (in cm) in IBA. When compared to MS media Urea supplementation with auxins showed the best results (Table 3). Plants with well-developed roots and shoots were transferred to coco peats under greenhouse conditions (Fig H). After primary hardening here well-developed plants were transferred to polythene bags containing a mixture of organic manure, sand and soil at a ratio of 1:1:2. (Fig I). Major steps from *in vitro* conditions to greenhouse conditions of the Malnad Rasbale plantlets micropropagation have been mentioned in figure 1 from Fig A to Fig I and percentage survivance of shoot tip of cv. Malnad Rasbale is shown in Fig 2 as a graphical representation. Out of 100 plants, 93% of plants survived in secondary hardening. Plantlets were well maintained in the greenhouse and were further transferred to field conditions (Kumar and Krishna 2015).

CONCLUSION

We hereby conclude that in our present investigation culture establishment of *Musa paradisica* cv. Malnad Rasbale was successful. This study transparently shows us the synergistic effect of urea supplemented media replacing potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3) as macronutrient basis gave the best results in the stages of initiation, multiplication, shoot development *and* root development. So urea can also be used as a substitute for nitrates source in *in vitro* conditions. The outcome of this investigation can be applicable for micropropagation and mass multiplication of endemic species of banana *Musa paradisica* cv. Malnad Rasbale and field evaluation studies can be carried out further.

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Author contributions

Ullas Prasanna S. designed the study, did the experiments, analyzed and interpreted the data *and* wrote the manuscript. Ravi Kumar S. helped in doing all experiments. Sachin S. Nayaka assisted in performing certain parts of the experiments. Ajith S. assisted in performing certain parts of the experiments. Prof. Krishna V. supervised and helped the whole work starting from design to manuscript reviewing. All authors revised and contributed to improving the quality of the manuscript.

Conflict of interest: None.

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