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Improvement of rice cultivar Kavuni for semi dwarf character by *in vitro* chemical mutation using Ethyl methyl sulphonate

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Abstract
The present study investigated the effect of an EMS treatment on the embryonic calli of Kavuni for the characters *viz*., late flowering, lodging, and long duration and improvement for these aspects will promote black rice cultivation. The experiment was conducted using Kavuni embryonic calli that showed 86.5% callus induction percentage and it was treated with EMS at 0.05%, 0.1%, 0.15% and 0.2% level along with control for mutagenesis to create dwarfism. The LC_{50} for EMS treatment based on callus survival was 0.10%. The maximum regeneration percentage of EMS treatment was 53.6% in the control and 0.1% EMS treatment recorded 40%. The lowest plant height of 20.2 cm in 0.05% EMS treatment. Nutritional analysis recorded the maximum protein content of 5.50% at 0.05% EMS and carbohydrate content was maximum of 54.56 at 0.05% EMS. The pigment *viz*., β-carotene, chlorophyll and anthocyanin were maximum of 0.02 µg/g, 2.25mg/g and 1.34 mg/g respectively at 0.05% EMS treatment. Total phenolics with the highest level of 28.5 mg/g was recorded at 0.05% EMS. The *in vitro* mutated rice Kavuni plantlet at 0.05% EMS concentration has improved characters and selection will be made in the subsequent generation for dwarf trait.

Keywords: Kavuni, *in vitro* mutation, LC_{50} value, regeneration, morphological and biochemical characterisation

Introduction
Rice is one of the most important cereal crop of the world meeting the dietary requirement of people living in tropics and subtropics. Asia accounts 90% of production and consumption of rice (Khush 2005) [18]. India has the world's largest area of rice cultivation in 42.5 million hectares and is the second largest producer next to China. Kavuni, a traditional brownish black rice variety is known for its anti-diabetic properties and is grown under limited area due to its long duration, poor tillering and photosensitive period. Kavuni grains are rich source of vitamins and antioxidants and its dark purple colour is primarily due to its anthocyanin content (Purwanto *et al.,* 2019) [31]. It is suitable for making porridge, dessert, traditional Chinese black rice cake, bread and noodles.

Black rice possesses significantly higher levels of β-carotene (46.2 µg/100g), lutein (221.6 µg/100g), total phenolics (28.8 µg/100g) and anti-oxidants activity. The health benefits of black rice are diverse and it prevents cancer and diabetes, reduces inflammation and body weight, improves liver and kidney health and builds immunity (Valarmathi *et al.*, 2015) [33]. A high antioxidant activity is contributed by phenolic compounds comprising of vanilic acid, caffeine acid, ferulic acid, p-coumaric acid, protocatechuic acid and 4-OH benzoic acid. In black rice the anthocyanin content ranged from 109.5 to 256.6 mg/100g.

Dwarfism is an agronomically important trait in breeding for resistance to damage by wind, rain and for stable high yields that increases the harvest index. Some of the varieties are very tall (>160cm) in stature and exhibit very late maturity (>150 days). Black rice and cultivars are hard to find currently and even nearing extinction. Black rice has the good flavour and aroma and is delicious. Due to the problem of tall stature, it is prone to lodging and late maturity with more fertilizer requirement.

Tissue culture technique offers a great potential for crop improvement through *in-vitro* mutagenesis by chemical mutagens. Mutation induction has become an established tool in crop improvement to improve cultivars for certain specific traits (Penna *et al.,* 2012) [30].

Radiation mutagenesis combined with *in vitro* culture has proven to be an useful method for the induction and selection of novel genetic variabilities (Jain 2010).

Brassinosteroids are involved in many morphological and physiological processes of rice, including the elongation and unrolling of leaves, development of tillers, root differentiation and reproductive growth (Mori *et al*., 2007) [25]. The reduction in plant height is enhanced from EMS concentration of 0.05% to 0.2%. The objective of this research is to develop early maturing Kavuni mutant with shorter plant crown and higher productivity by chemical mutation using ethyl methane sulphonate (EMS). Also, identification of Kavuni dwarf mutant lines with good combination of nutritional qualities will be used for future breeding programmes.

Materials and Methods

The research was conducted at Agricultural College and Research Institute Killikulam. Healthy, pure, uniform and dry seeds of Kavuni rice were inoculated for callus induction in MS media (Murashige and Skoog, 1962) [26] as described by (Amaravel 2019) [2]. Also the embryogenic calli formed was treated with different EMS concentrations of 0.05%, 0.1%, 0.15% and 0.2% as described by Venkateshan, 2021 [34]. After two weeks of inoculation callus induction percentage was calculated and survival percentage was recorded after EMS treatment. Relative growth rate and RDR were calculated after three times of sub culturing.

$$\text{Callus induction percentage} = \frac{\text{Number of seeds that produced callus}}{\text{Total seeds cultured (100)}} \text{X } 100$$

$$\text{Survival percentage} = \frac{\text{Number of calli that survivied}}{\text{Total number of gamma irradiated calli}} \text{X } 100$$

Relative growth rate (RGR %) = $(W_n – W_o / W_o) \times 100$

Where

W_n Weight of the calli at the end of the experiment

W_o Weight of the calli at the start of the experiment

$$\text{Relative differentiation rate (RDR \%)} = \frac{\text{No. of survived calli in treatment}}{\text{No. of survived calli in control}} \text{ X } 100$$

The irradiated calli after sub culturing were inoculated in regeneration media consisting of BAP 3mg/l, NAA 1mg/l, maltose 30g/l and clerigel 3g/l (Amaravel *et al.*, 2019) [2]. The regeneration percentage of calli at various EMS treatments was calculated.

$$\text{Regeneration percentage} = \frac{\text{Number of calli that produced green shoot}}{\text{Total number of calli transferred to regeneration media}} \text{ X } 100$$

The *in vitro* regenerated shoots were transferred for *in vitro* rooting to half MS medium without hormones for two weeks and the plantlets were transferred to greenhouse condition for two weeks and subsequent to shade net house for hardening.

After three weeks of hardening, biochemical analysis was done in Kavuni rice mutant plantlets. Anthocyanin pigment content (Pedro *et al.*, 2016) [29], chlorophyll content (Khalil *et al.*, 2018) [17], β-carotene, protein content (Lowry *et al*., 1951) [23] and carbohydrate content (Hodge *et al.*, 1962) [13] were analysed. The LC_{50} values were calculated based on the survival percentage and relative differentiation rate of calli using curve expert 1.4 programme. The experimental data sets observed were analysed descriptively using the analysis of variance at 5% confidence level using DMRT analysis.

Results and Discussion

Callus induction percentage

In rice tissue culture, the callus induction mainly depends on the media composition, genotype of the mother plant, explants type and plant growth regulators (Khaleda and Forkan, 2006). The maximum callus induction was observed in MS Medium (Murashige and Skoog, 1962) [26] supplemented with 2,4- D 2mg/l, kinetin 0.5mg/l and surcrose 30g/l. The callus induction percentage was observed as 86.5% in the cultivar, Kavuni (Table 1.). According to Biswas and Mandal (2007) [4], MS Medium supplemented with 2,4-D and kinetin had a significant effect on callus induction percentage.

Paul and Roychoudhry (2019) [8] observed callus induction percentage in aromatic rice cultivars that ranged from 63.19% to 98.33% in eight rice cultivars and similar results by Bolivar *et al*. (2018) [5] in coffee. Luan *et al*. (2007) [24] in potato found mutagenic effect in embryonic calli when treated with different concentrations of EMS

In vitro mutagenesis of rice calli for dwarfism

Survival percentage, Relative Growth Rate and Relative Differentiation Rate of EMS treated calli

A gradual decrease in the survival percentage of calli was observed when exposed to an increasing concentration of EMS from 0.05% to 0.2% when compared to that of control (Table 2.). In the EMS treated calli, the maximum survival percentage was observed as 86.6% in control followed by 50%, 36.67%, 26.68% and 13.33% in 0.05%, 0.1%, 0.15% and 0.2% EMS concentrations of respectively. Acanda *et al*. (2014) reported a reduction in the embryonic calli survival rate at 10mM EMS concentration when compared with 1mM and 3mM EMS treatments in grapevine and similar decreasing survival percentage of calli was reported by Kumar *et al*. (2010) in rough lemon, Ekanayaka *et al*. (2016) in rice and Khalil *et al*. (2018) [17] in sugarcane. According to Das *et al*. (2010), a reduction in survival percentage depends on the EMS concentration and duration of the treatment in *Withania.*

The relative growth rate of the callus showed a linear decrease in the growth of the callus as the EMS concentration increases from 0.05% to 0.2% EMS and significant difference was observed among the various EMS concentrations. The highest relative growth rate of calli in the variety Kavuni was observed as 1.64 in control without EMS treatment followed by 0.86 at 0.05% EMS, 0.79 at 0.1% EMS, 0.65 at 0.15% EMS and 0.55 at 0.2% EMS. Similarly, Kumar *et al*. (2011) [20] observed striking differences not only in the callus induction but also in the weight of the calli. Pauli *et al*. (2019) [8] stated that EMS mutagens showed a significant impact on the callus growth by increasing EMS concentrations when compared to the untreated calli and resulted in callus necrosis at high EMS concentrations (Koch *et al*., 2012) [19].

The relative differentiation rate is calculated for fixation of optimal dose for maximum heritable variation with minimum lethality. Among the various EMS treatments, the Kavuni embryogenic calli showed the relative differentiation rate of 100% in control followed by 78.57%, 45.71%, 21.42% and 15.51% at 0.05%, 0.1%, 0.15% and 0.2% EMS concentrations respectively.

Determination of LC_{50} of EMS treated calli

Newly proliferating embryonic calli was sensitive to the EMS treatment. The survival percentage of EMS treated embryonic calli decreased with increasing EMS concentrations. The LC_{50} value based on survival percentage was 0.10% (Table 2.) Ge

*et al. (*2015) reported the lethal concentration of embryonic calli of sweet orange as 0.15% EMS. Gadakh *et al*. (2015) [10] reported LD_{50} of sugarcane varieties CO 99004 and CO94012 embryonic calli as 0.05% EMS. The LD_{50} in rice cell suspension culture was 0.4% (Chen *et al*., 2013) [6]. Dong *et al*. (2017) reported the optimal EMS concentration for rice varieties IR 231 and MR 219 as 0.05% and 0.06% respectively and showed high sensitivity to EMS.

Regeneration response of EMS treated calli

The regeneration percentage decreased with increase in EMS concentration. In EMS treated calli, the maximum regeneration percentage observed was 53.6% in control and minimum percentage was 6.6% at 0.2% EMS followed by 20% at 0.15% EMS, 26% at 0.1% EMS and 40% at 0.05% EMS as shown in Table 3. Similarly, Joong and Lee (2002) stated that at high EMS concentration the regeneration frequency was reduced. The abnormal plants with chlorophyll deficiency, weak culm and albino plants with no root formation were observed. Similar studies by Rakshana *et al*. (2019) [32] reported the maximum regeneration frequency of 80% with average of 12 shoots per calli in NB media of 3mg/l 6-BA, 0.5mg/l kinetin, 3% maltose and 0.3 g/l glutamine. Venkateshan (2021) [34] reported a reduced regeneration frequency of 5% at 0.2% EMS in Kalinga and 2% at 0.3% EMS in Kuliyadichan.

Plant height of *in vitro* EMS mutated Kavuni plantlets

Plant height is an important character in plant breeding improvement because it is closely associated with effective utilisation of assimilates to result in the formation of improved plant products. Mutants produce dwarf stature, semi dwarf stature, early maturity, increased tillering, high grain yield and other desirable traits.

In EMS treatment of embryogenic calli and subsequent regeneration, the plant height was observed as 22.5cm in control followed by 22.4cm in 0.15% EMS, 21.8cm in 0.2% EMS, 21.4cm in 0.1% EMS and 20.2cm in 0.05% EMS. Similar findings were reported by Nasri *et al*. (2021) [27] in chrysanthemum cultivars treated with ethyl methane sulphonate of 0%, 0.125%, 0.25%, 0.5% that showed a novel variation in leaf colour and shape, plant height, days to flowering and inflorescence head size. Arici *et al*. (2018) [3] stated that *in vitro* culture of Alanson potatoes when treated with a different EMS concentrations of 20 mM, 50 mM, 75 mM and100 mM resulted in the plant height of 7.65cm at 100 mM followed by 7.17 cm at 20mM.

Biochemical and nutritional evaluation of *in vitro* mutated plantlets of Kavuni

Protein and Carbohydrate content

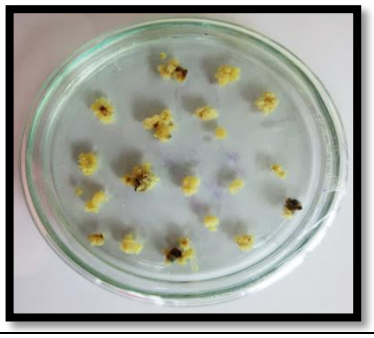
Among all the EMS treatments, the highest protein content was observed as 5.50% at 0.05% EMS followed by 5.14% at 0.2%, 5.02% at 0.1%, 4.56% in 0.15% and 4.22% in control. The carbohydrate content ranged from the maximum of 54.56% at 0.05% EMS and minimum of 48.22% in control followed by 49.06%, 51.94% and 52.78% at 0.15%, 0.1% and 0.2% EMS respectively. Gunasekaran *et al*. (2015) observed a reduced protein content of 32% at 0.2% EMS treatment when compared with control with a protein content of 34.58%.

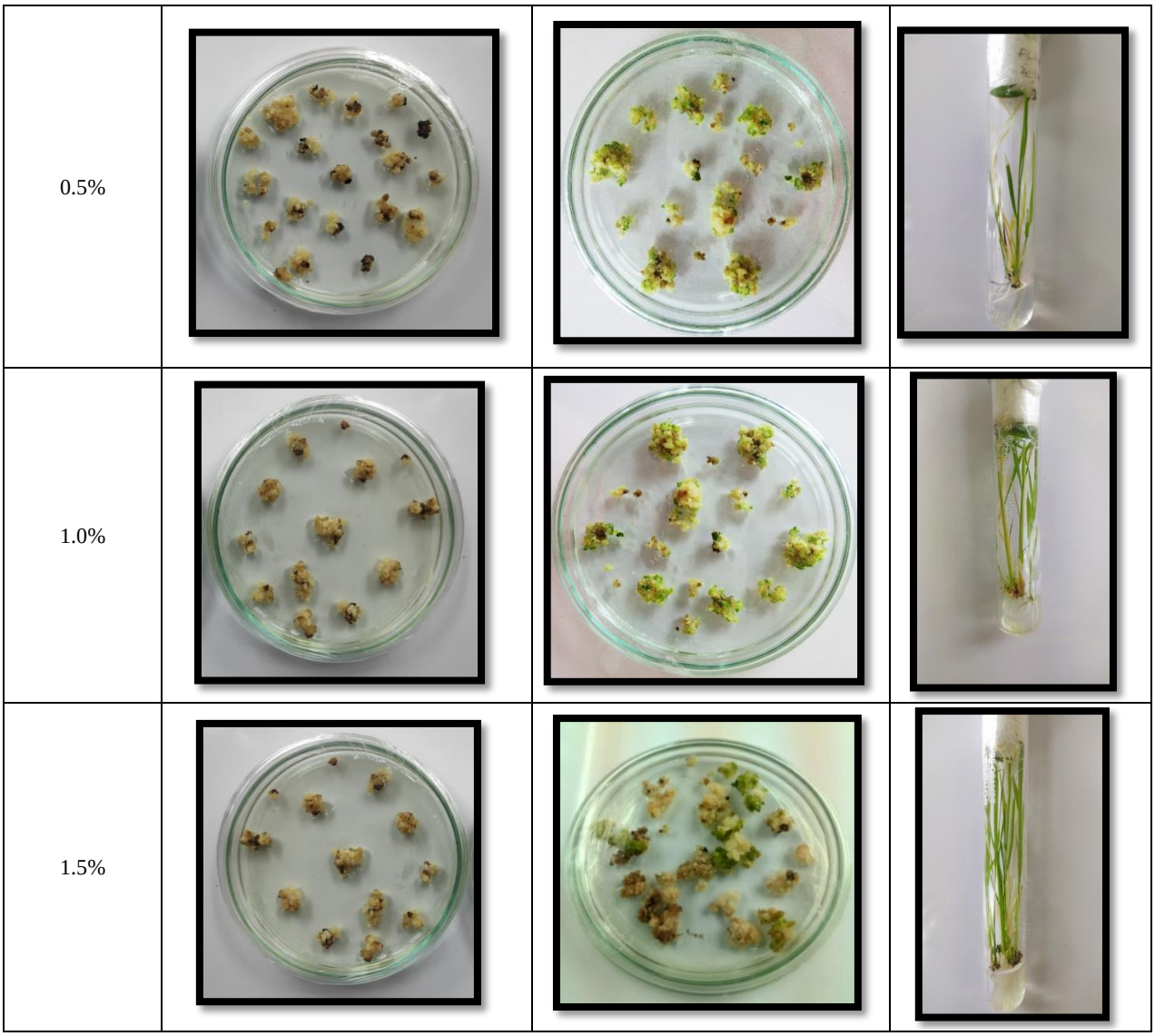
β - Carotene, Chlorophyll and Anthocyanin content

The maximum β Carotene was observed as 0.02µg/g at 0.05% EMS and the minimum of 0.012 µg/g in 0.15% EMS followed by 0.014 µg/g in control, 0.015 µg/g at 0.1% and 0.017 µg/g at 0.2% EMS concentrations respectively. The maximum chlorophyll content was observed as 2.25 mg/g at 0.05% EMS and minimum of 1.54 mg/g at 0.15% EMS followed by 1.60 mg/g in control, 1.88 mg/g at 0.1% EMS and 2.02 mg/g at 0.2% EMS concentration. The maximum anthocyanin content was observed as 1.34 mg/g at 0.05% EMS and minimum of 1.21 mg/g at 0.15%EMS followed by 1.25 mg/g in control, 1.26 mg/g at 0.1% EMS and 1.30 mg/g at 0.2% EMS concentration respectively. Valarmathi *et al*. (2017) [32] reported that EMS concentration of 0.3% increased chlorophyll and carbohydrate content and decreased in other EMS concentrations. According to Kumar *et al*. (2018) [21] the photosynthetic pigments showed a significant variation in the EMS treated and control set of coriander.

Phenolics

The maximum phenolics observed was 28.5 mg/g at 0.05% EMS and minimum of 18.9 mg/g at 0.15% EMS followed by 20.2mg/g in control, 21.6 mg/g at 0.1% EMS and 24.4 mg/g at 0.2% EMS concentration. It is hold up with the results of Valarmathi *et al*. (2017) [32].

Treatment	EMS treated calli	Regenerated calli	Regenerated plants
Control			



Treatment	EMS treated calli	Regenerated calli	Regenerated plants

Table 1: Callus induction percentage in the rice cultivar Kavuni

Variety	No of seeds inoculated	Embryonic callus from seeds	Callus induction (%)
Kavuni	400	346	86.5

Table 2: Survival percentage, Relative growth rate, Relative differentiation rate and LC_{50} value of EMS treated embryogenic calli.

EMS Concentration (%)	Survival percentage (%)	Relative growth rate RGR	Relative differentiation rate RDR (%)	LC_{50}
Control	86.66	$1.64 \pm 0.04_{a}$	100	0.10%
0.05%	50.00	$0.86 \pm 0.24_{bc}$	78.57	
0.1%	36.67	$0.79 \pm 0.05_{cd}$	45.71	
0.15%	26.68	$0.65 \pm 0.05_{d}$	21.42	
0.2%	13.33	$0.55 \pm 0.07_{c}$	15.51	

Relative growth rate values are analysed by DMRT Analysis. Data represented by Mean ± SE.

Table 3: Regeneration percentage of *in vitro* survived calli after EMS treatment in rice variety Kavuni

EMS treatment (%)	No. of calli inoculated	No. of calli that turned green	No. of calli that regenerated	Regeneration percentage (%)
Control	30	28	15	53.6%
0.05%	30	20	12	40.0%
0.1%	30	14	8	26.0%
0.15%	30	10	6	20.0%
0.2%	30	5	2	6.6%

Table 4: Morphological and biochemical parameters of *in vitro* EMS mutated Kavuni plantlets.

EMS treatments (%)	*In-vitro* Plantlet Height (cm)	Protein content (%)	Carbohydrate content (%)	β – carotene (µg/g)	Chlorophyll (mg/g)	Anthocyanin (mg/g)	Phenolics (mg/g)
Control	22.5	4.22	48.22	0.014	1.60	1.25	20.2
0.05%	20.2	5.50	54.56	0.020	2.25	1.34	28.5
0.1%	21.4	5.02	51.94	0.015	1.88	1.26	21.6
0.15%	22.4	4.56	49.06	0.012	1.54	1.21	18.9
0.2%	21.8	5.14	52.78	0.017	2.02	1.30	24.4

Conclusion

The overall study on the effect of EMS treatment on the embryonic calli decreased the survival percentage, relative growth rate and relative differentiation rate was decreased linearly with increasing concentration of EMS from 0.05% to 0.2%. The highest regeneration percentage was observed in at 0.05% EMS concentration. Further generation will be studied in order to select the stable mutant for dwarfism in rice variety, Kavuni.

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