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CALLUS INDUCTION, REGENERATION OF RICE VARIETY BRRI dhan29 AND DETERMINATION OF LETHAL DOSE OF ETHYL METHANESULFONATE

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Abstract

Rice is an essential meal for a large section of the world's population including Bangladesh. BRRI dhan29 is known as an excellent rice variety in Bangladesh but it is now susceptible to several diseases and pests, which might reduce rice yields. Therefore, it becomes necessary to explore and optimize different approach with the aim of enhancing yield, diseases resistance and overall genetic variability of this important rice variety. Ethyl methanesulfonate (EMS) produces a large number of non-lethal point mutations by alkylates guanine bases and leads to mispairing-alkylated guanine pairs with thiamine instead of cytosine. The LD_{50} of EMS is frequently used as a general indicator of a substance's acute toxicity and a lower LD_{50} is indicative of increased toxicity. This study aimed to develop a comprehensive protocol for callus induction, regeneration for the BRRI dhan29 rice and to determine the LD50% of EMS on callus survival for further genetic improvement. Mature embryos were used to induce callus by utilizing different concentrations (0.00, 0.5, 1.00, 1.50, 2.00, 2.50, 3.00 and 3.50 mg/L) of 2,4-Dichlorophenoxyactic acid (2, 4-D), while callus regeneration was studied using varying combine concentrations of 1-naphthaleneacetic acid (NAA: 0, 5, 10 and 15 µg/L) and kinetin (0.00, 1.00, 2.00 and 3.00 ml/L). The optimal concentrations for callus induction was 2.0 mg/L 2, 4-D and a combination of 10 µg/L NAA and 2.0 mg/L kinetin were identified for efficient callus regeneration. The LD_{50} of EMS on BRRI dhan29 calli was 0.31%, indicating its potential mutagen for creating genetic variations. Regenerated plants were successfully acclimatized at field conditions. These findings might provide valuable insights for tissue culture-based studies and genetic improvement efforts for the BRRI dhan29.

Key words: *In vitro* regeneration, Acclimatization, Phytohormones, Rice

Introduction

Rice, being a crucial cereal crop and a fundamental food source for more than half of the global population, is primarily cultivated in emerging Asian countries (Muthayya *et al.*, 2014). Most of the rice production takes place in Asian nations, where it is consumed by approximately half of the world's population (Prasad *et al*., 2017). Indica rice, which is long-grained and grown in tropical regions, constitutes the largest portion of cultivated rice among the two main subspecies (*indica* and *japonica*) (Shi *et al.*, 2022). Establishing an efficient and widely utilized tissue culture system for rice holds significant potential for accelerating rice varietal development programs and effectively employing genetic mutation technology to enhance cultivars, particularly in terms of combating biotic and abiotic stress and growth parameters.

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The *in vitro* culture system is an exciting area of research because it has a high potential for successfully regenerating fertile rice plants from various plant parts (Ahmad *et al*., 2016). For genetically modification and improvement of rice varieties, it is crucial to have a high percentage of embryogenic calli that can be regenerated (Paul and Roychoudhury, 2019). However, many indica rice types are challenging to manipulate *in vitro* due to their poor ability to form callus, proliferation and regeneration. Researchers have been working to identify suitable plant hormones and create optimal *in vitro* conditions to induce the formation of embryogenic callus.

Ethyl methanesulfonate (EMS) and sodium azide (SA) are the primary chemical mutagens used to induce mutations in plants (Panda *et al*., 2022). Their effectiveness depends on the concentration and duration of treatment (Lee *et al*., 2017). EMS is the most commonly used chemical mutagen in plants because of its functional potentiality and easy handaling. EMS alkylates guanine bases and leads to mispairing-alkylated G pairs with T instead of C, resulting in primarily G/C- to-A/T transitions (Bhat *et al.*, 2007). Since EMS produces a large number (genome-wide) of non-lethal point mutations, a relatively small mutant population is sufficient to saturate the genome with mutations. The lethal dose, LD_{50} (lethal dose, 50%) of a toxin, radiation, or pathogen is the dose required to kill half the members of a tested population after a specified test duration. The LD_{50} are frequently used as a general indicator of a substance's acute toxicity and a lower LD_{50} is indicative of increased toxicity.

These mutagens have been successfully used to induce mutations in various crops such as barley, wheat, rice, oat, tobacco, corn, and potato (Yadav *et al.,* 2021). Researchers have made numerous attempts in rice to identify the most effective mutagenic treatment for inducing mutations related to plant height, chlorophyll content, seed germination enhancement, and root improvement (Viana *et al.,* 2019).

Studying the cellular structure of rice embryogenic callus at different stages is crucial to understand its internal organization (Ma *et al.,* 2015). Researchers have identified two distinct clusters of cells, embryogenic and non-embryogenic, during the process of somatic embryogenesis in a rice cultivar (Ming *et al., 2019).* By manipulating the appropriate culture medium and conditions, it is possible to obtain embryogenic callus with a high regeneration capacity (Phillips and Garda, 2019). This is important for both traditional breeding and genetic transformation purposes to enhance rice through genetic improvement. The BRRI dhan29, which is highly popular rice variety in Bangladesh, has become vulnerable to various diseases (Hossain *et al.,* 2017). In order to enhance its resistance against these diseases through genetic modification either by somaclonal variations or genetic modification studies, it is necessary to develop efficient regeneration protocol. The objectives of this study were to establish a protocol for callus induction and regeneration for BRRI dhan29, and to determine the LD50 (lethal dose 50%) of EMS (ethyl methanesulfonate) on the callus of BRRI dhan29.

Method and Material

This study was conducted at the Biotechnology Lab BINA. The experimental design employed was a completely randomized design (CRD), with five replications.

Explants sterilization

For the preparation of explant, healthy and disease-free rice grains were chosen. After keeping fresh seeds to 45^{o}C for 12 hours and removing the husk, intact and white seeds were selected. Fresh and mature embryo were rinse with sterile distilled water for three times, followed by a treated with 70% ethanol. The seeds were then washed and soaked in a solution of 3% sodium hypochlorite with tween-20 for 30 minutes. After multiple washes with sterile water, the seeds were air-dried on Whatman filter paper. This process ensured the selection of suitable seeds for callus induction in this study (Khan *et al.*, 2021).

Callus induction

For initiation and maintaining callus Murashige and Skoog complete media (Murashige and Skoog, 1962) was used with the supplementation of 2,4-D and phytohormones. The media composition was based on Murashige and Skoog complete media supplemented with L-proline, casamino acid, sucrose, and varying doses of 2,4-D (Khan *et al.*, 2021). Gelatin was used to solidify the media. Media was prepared freshly, then sterilized and dispensed into Petri dishes. Callus initiation was accomplished by placing mature embryos on the medium, with the seed endosperm deeply embedded. Prior to seeding, the medium was punctured with sterile tweezers, and after seed plating the plates were sealed with parafilm. The cultures were then incubated under continuous light at 28^{O}C for ten days, and data on callus induction was recorded thereafter.

Application of EMS and callus regeneration

Different volume percentages of EMS were applied on ten-days-old calli as a chemical mutagen for a duration of two hours, followed by cultivation in liquid regeneration medium comprising kinetin, NAA, casamino acid, sucrose, and sorbitol (Khan *et al.*, 2021). Incubation with shaking was carried out at 28^{0}C for 48 hours. Regeneration media, based on the formulation of Murashige and Skoog, were prepared and used to support the growth of ten-day-old embryogenic calli. The cultures were maintained at 28^{0}C with an 18-hour photoperiod for 30 days, with regular media changes every 10 days. After 21 days, data on callus regeneration ability were recorded.

Root initiation and acclimatization

Rooting media was prepared according to Murashige and Skoog formulation and used to initiate root growth without hormones (Khan *et al.*, 2021). The regenerated plantlets were transferred to the rooting media and maintained under controlled conditions (28^{O}C

with an 18-hour photoperiod) for another 30 days. Following that, the plantlets were acclimatized by removing them from the media, washing them thoroughly and transferring them to plastic pots filled with vermiculite. The pots were covered with polyethene for two weeks to prevent desiccation before gradually transitioning the plants to growth room conditions. Eventually, the plantlets were transferred to field conditions for further growth and development until reaching maturity

Data analysis

The boxplot analysis for callus induction and callus regeneration was conducted using Tukey's test in RStudio software, utilizing the "ggplot2" and "multcompView" packages. The boxplot provided information about the normality of the data and facilitated the comparison of different treatment groups. On the other hand, the regression graph for determining the LD50% was generated using Microsoft Excel.

Result and discussion

Callus Induction

The *in vitro* induction of callus was investigated using mature embryos of BRRI dhan29 rice variety. Different concentrations of 2, 4-D (1.0, 2.0, 3.0, and 4.0 mg/L) were added to the media, while a control group without 2,4-D was also included as control. The callus induction percentage of BRRI dhan29 at various 2,4-D concentrations is illustrated in Fig. 1, along with other comparisons denoted by letters. The box in the graph symbolises the distribution of data; the black dot is the data mean and the black line in the box is the median. The callus induction efficiency of BRRI dhan29 ranged from 9% to 92% for different 2,4-D concentrations (Fig. 1). The control group, without any 2, 4-D treatment, did not exhibit any callus development. Among the various concentrations tested, the highest callus induction ability (87%) was observed at T_4 (2 mg/L 2,4-D) treatment. This result suggesting that 2,4-D treatment is essential for callus initiation and an optimal concentration of 2,4-D promotes efficient callus formation in BRRI dhan29. The 2,4-D induces callus formation by disrupting the hormonal balance in plant tissues through its synthetic auxin activity (Garcia *et al.,* 2019). However, it was observed that when the 2,4-D concentration dose exceeded 2.0 mg/L, the frequency of callus induction was decreased. This finding indicated that although lower concentration is essential for callus initiations but higher concentrations of 2,4-D have an inhibitory effect on callus induction. It was happened because higher concentrations of 2,4-D create an inhibitory effect on callus induction due to the cytotoxicity of the herbicide, which can negatively impact on cell viability and growth, thereby suppressing the formation of callus tissue (Zhang *et al.,* 2007). Many investigations suggested that the presence of synthetic auxin 2,4-D was an essential component in the effective induction of rice callus (Shweta *et al.,* 2020; Poeaim *et al.,* 2016; Ahmad *et al*., 2016).

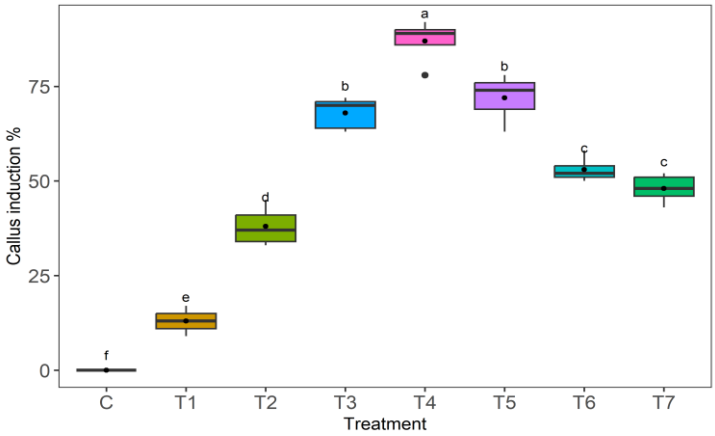

Fig. 1: The boxplot graph shows impact of various concentrations of 2,4-D on callus induction in BRRI dhan29. Here, C=0.0 mg/L, T1 = 0.5 mg/L, T2 = 1.0 mg/L, T3 = 1.5 mg/L, T4 = 2.0 mg/L, T5 = 2.5 mg/L, T6 = 3.0 mg/L and T7= 3.5 mg/L of 2,4-Dichlorophenoxyacetic acid (2,4-D)

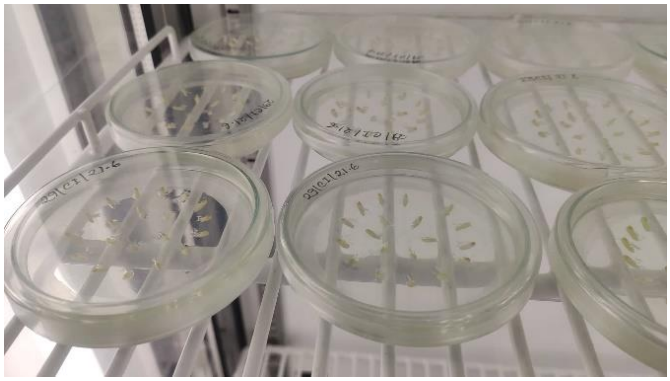

Fig. 2: Mature endosperm of BRRI dhan29 plating in callus induction media

Callus regeneration

Calli obtained from MS media supplemented with 2.0 mg/L 2,4-D were evaluated for their ability to regenerate into plants. For regeneration, the impact of different concentrations of kinetin (0, 1, 2, and 3 mL/L) and NAA (0, 5, 10, and 15 µg/L) on plant regeneration (Fig. 3) were evaluated. Fig. 3 depicts the callus regeneration percentage of BRRI dhan29 at various combinations of kinetin and NAA. The figure also includes comparisons of the treatments, denoted by letters. The graphical representation shows the distribution of the data using a box, with the mean indicated by a black dot and the median represented by a black line inside the box. Notably, all treatments exhibited significant effects on plant regeneration compared to the control group. Among the treatments, treatment T_5 (10 µg/L NAA + 2 mg/L kinetin) demonstrated the highest regeneration ability, with a frequency of 74%. The combination of NAA and kinetin plays a crucial role in promoting plant regeneration. The NAA acts as an auxin, promoting cell division and callus

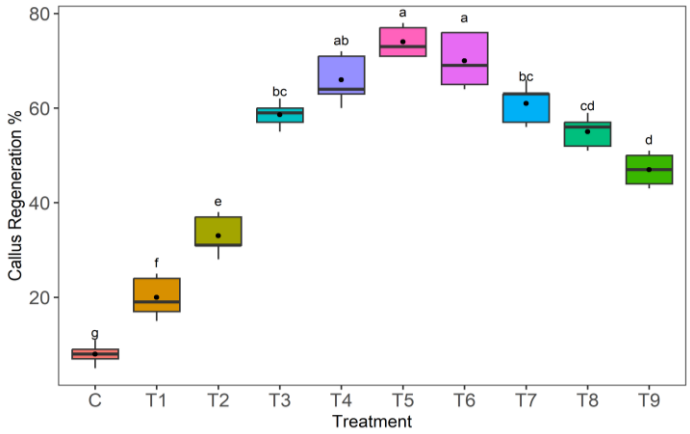

Fig. 3: Boxplot graph shows the callus regeneration percentage of BRRI dhan29 at various combinations of kinetin and NAA. Here, combination of Kinetin+NAA are, C= 0 ml/L+0 µg/L, T1= 1 ml/L+5 µg/L, T2= 1 ml/L+10 µg/L, T3= 1 ml/L+15 µg/L, T4= 2 ml/L+5 µg/L, T5= 2 ml/L+10 µg/L, T6= 2 ml/L+15 µg/L, T7= 3 ml/L+5 µg/L, T8= 3 ml/L+10 µg/L and T9= 3 ml/L+15 µg/L

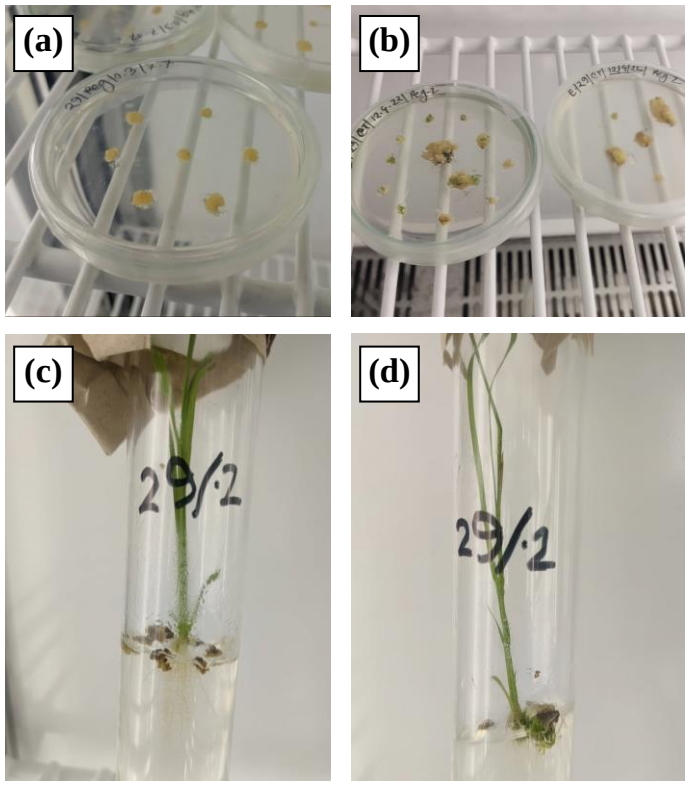

Fig. 4: a and b shows the calli cultured in regeneration media; c and d showed the plantlets cultured in rooting media.

formation, while kinetin acts as a cytokinin, stimulating shoot induction and growth (Sieberer *et al*., 2003; Martins *et al.*, 2022). The combined application of NAA and kinetin provides optimal hormonal balance, enhancing the efficiency of plant regeneration in tissue culture protocols. The ideal concentration for optimal plant regeneration of BRRI dhan29 was 10 µg/L NAA and 2.0 mg/L kinetin in MS media.

Acclimatization

Acclimatization is an important step for obtaining plantlets from regenerated tissue. During the regeneration process, the *in vitro* grown shoots exhibited robust growth, displaying a healthy and vibrant green color. The shoots were carefully transferred to a 100 percent vermiculite substrate and placed in a growth chamber covering with polybags set at 25°C with a 16-hour photoperiod. The growth chamber provided a cool white fluorescent light source, delivering an approximate light intensity of 30-40 mol/m^2s. Impressively, around 75 percent of the shoots were successfully acclimated and demonstrated high survival rates. Within a span of three weeks, the transplanted shoots exhibited vigorous root growth, indicating successful establishment and adaptation to the new environment. Following this stage, the shoots were further transplanted into larger pots and gradually exposed to normal environmental conditions. Throughout the acclimatization period, the rooted and shoots displayed active growth and did not exhibit any signs of stress or morphological abnormalities. These promising results affirm the successful acclimatization of the shoots, indicating their readiness for further growth and development in natural conditions.

Fig. 5: Acclimatization of plantlets obtained from BRRI dhan29 regeneration in the culture chamber using vermiculite.

Determination of LD_{50}%

The LD50% of EMS (ethyl methanesulfonate) for BRRI dhan29 rice genotype was determined by exposing ten-day old calli to different concentrations (0, 0.05, 0.1, 5.25, 0.35, 0.35, 0.35, 0.4% and 0.45%) of EMS for 120 minutes. The LD_{50}% value of EMS was found to be 0.31% for the given duration (Fig. 6). As the concentration of EMS increased, the survival rate of the calli decreased. The untreated calli showed a survival rate of over 90%, while the calli exposed to 0.45% EMS exhibited the lowest survival rate of 21%. These

findings demonstrate the dose-dependent effect of EMS on callus survival, with higher concentrations leading to reduced survival rates. The EMS is a potent mutagen that induces genetic mutations by alkylating DNA (Snyman *et al.*, 2021). However, at higher concentrations, EMS can exert cytotoxic effects on cells, causing cell death and reducing the viability of rice calli. Decreasing rate of survival in our study may be attributed to the increased DNA damage and cellular stress caused by excessive EMS exposure.

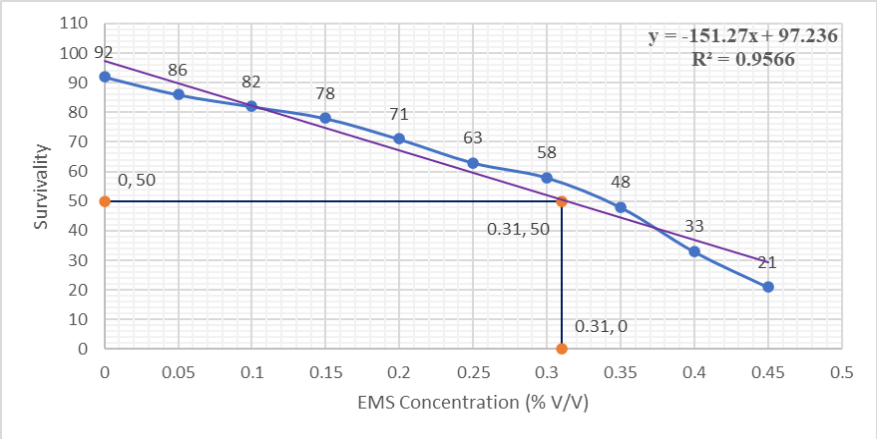

Fig. 6: Survival rate of BRRI dhan29 rice calli exposed to different concentrations of EMS (ethyl methanesulfonate) for 120 minutes

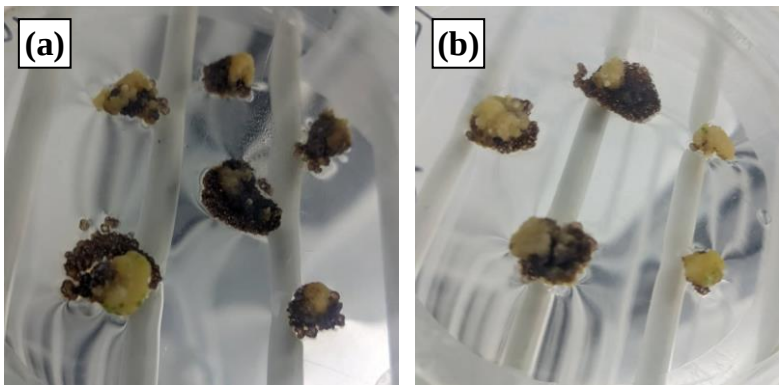

Fig. 7: a and b BRRI dhan29 rice calli exposed to increasing concentrations of EMS for 120 minutes, demonstrating reduced viability and cell death

Conclusion

In conclusion, this research successfully established a protocol for callus induction and regeneration of the BRRI dhan29 rice variety. Optimal concentrations of 2,4-D (2.0 mg/L) and a combination of NAA (10 µg/L) and kinetin (2.0 mg/L) were identified for efficient callus induction and plant regeneration, respectively. These findings provide valuable insights for future tissue culture-based studies and genetic improvement programs

in BRRI dhan29. Additionally, the LD_{50}% of EMS on BRRI dhan29 calli was determined to be 0.31%, indicating its potential as a mutagen for inducing genetic variations. These results contribute to our understanding of in vitro manipulation techniques and offer practical applications for crop improvement programs in BRRI dhan29.

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