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SYNERGISTIC EFFECT OF CASEIN HYDROLYSATE AND 2,4-D ON IN VITRO CALLOGENESIS AND SUBSEQUENT REGENERATION IN RICE (ORYZA SATIVA L.)

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ABSTRACT

Even after three decades of invention, plastid transformation has been very difficult especially for monocot crops. Difficulty in attaining, efficient *in vitro* regeneration is one of the many factors affecting plastid transformation. Hence, present study was planned to investigate the impact of 2,4-D and casein hydolysate on *in vitro* callogenesis and subsequent regeneration of different Pakistani rice cultivars. For this purpose, mature seeds of six Pakistani genotypes IR-6, KSK-133, KSK-434, KS-282, PK-386 and Super Basmati were used. Scutellum from mature rice seeds were incubated on MS medium augmented with 2,4-D (1-5mg/L) to study callogenesis and subsequent regeneration response. Maximum callus formation was obtained in variety IR-6 (2mg/L 2,4-D) and callus size was found increasing with increasing 2,4-D concentration. Later, synergistic effect of casein hydrolysate and 2,4-D was investigated in two steps. Firstly, effect of 2,4-D in combination with 6mg/L casein hydrolyste was observed. Maximum callogenesis was found in IR-6 at 2mg/L 2,4-D and 6mg/L casein hydrolysate. Secondly, effect of casein hydrolysate (4-8mg/L) with 2,4-D 2mg/L was examined and maximum callus formation was found in variety IR-6 (casein hydrolysate 4mg/L + 2,4-D 2mg/L). Then, calli were shifted to different combinations of Plant Growth Regulators (PGRs) i.e. NAA + Kinetin and IBA + BAP to optimize regeneration. Differential response of varieties was examined. Maximum regeneration was observed in PK-386 (2.5 plantlets/callus).

***Keywords*:** Callus induction, rice, 2,4-D, NAA, Kintein.

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1. INTRODUCTION

Rice being the 3rd most significant crop after wheat and cotton (Abbas 2022) and one of the most important export items of Pakistan. Rice is the diet of more than 50% of the global population, and in Asia, rice alone accounts for about 70% of the total dietary calories consumed daily. With the world population projected to reach 8.9 billion by 2030, it will need to increase rice production by more than 40% to fulfil the dietary requirements. Rice varieties being grown in Pakistan, especially 'Basmati', are known worldwide for their flavour, aroma and non-sticky extra-long grain (Akhter and Haider 2020). Increased rice production therefore contributes to world hunger eradication, poverty alleviation, food security and economic development. Many efforts have been made to develop high-yielding cultivars with better nutritional quality through traditional and mutation breeding, extensive crossing, and somatic clonal variation (Rai 2022). Despite all these efforts, Pakistan's average rice yield per unit area is much lower and even lower than many neighboring countries (Li et al. 2019). This has created a need in Pakistan to improve commercial varieties through biotechnology and genetic transformation.

Chloroplast transformation's just at its beginning stage. Applications of chloroplast genetic engineering are limited to a very small number of plant species i.e. tobacco (Wang 2022), potato (del Mar Martínez-Prada et al., 2021), tomato (Yang et al., 2020) and rapeseed. Furthermore, there are no reports of transforming chloroplasts to obtain fertile monocotyledonous plants (Khan and Maliga, 1999; Khan, 2007). Hence, it is necessary to establish chloroplast transformation protocols in many economically important crops. Rice is the most important crop grown worldwide. We are developing an efficient chloroplast gene expression system in rice. Due to its unique advantages, plastid transformation is now becoming a promising research topic for improving rice genetics to obtain resistant traits.

Two types of PGRs especially cytokinins and auxins, are important in plant growth under *in vitro* conditions. The application of PGRs has been made to induce distinct processes, such as embryogenesis, organogenesis and

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rhizome as well as microtuber formation from *in vitro* cultured tissues and cells (Liang et al., 2021). By virtue of the boundless advantages of plant transformation technology, *in vitro* methods of plant tissue culture have developed tremendously in the past decades. The employment of meristematic tissues forms the foundation of tissue culture protocols. The regenerative response of important cultivars is generally poor. Hence, for the effective application of plant tissue culture for crop improvement requires a proficient method of callus induction and *in vitro* regeneration.

The objective of the study was to realize an efficient in vitro regeneration procedure for rice plants using callus derived from the adult seed coat and using it in biotechnology to develop agronomic traits. desired learning by genetic engineering.

2. MATERIALS AND METHODS

2.1. Plant Materials

As an explant source for the callogenesis from scutellum, mature seeds were used in this study. The Rice Research Institute at Kala Shah Kaku, Lahore, provided seeds for the various rice varieties. Physical characteristics were used to pick ripe, healthy seeds, and they were manually dehusked.

2.2. Media Composition

Surface sterilized mature seed were cultured on MS media containing 30g/L $C_{12}H_{22}O_{11}$, 4.33g/L MS salt, 100 mg/L myo-inositol, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxin and 0.1 mg/L thymine HCl gelled with 3.66 g/L phytagel. Additionally, different concentrations of 2,4-D (1.0-5.0 mg/L) and casein hydrolysate (4-8 g/L) alone and in combination for active callogenesis and improved *in vitro* regeneration.

2.3. Planting Seeds on Petri Dishes

Seeds were stored in a petri dish on autoclaved filter paper after explants were surface sterilised. With sterilised forceps, the seeds were injected onto the culture plates after the water had been wiped from their surface.

2.4. Initiation of a Callus

To induce callus growth, various 2,4-D doses were utilised. For the purpose of inducing calluses, MS (Murashige & Skoog, 1962) basal medium supplemented with various 2,4-D (1.0-5.0 mg/L) and casein hydrolysate (4-8g/L) concentrations were created. The medium's pH was adjusted to 5.8 and phytagel was used to solidify it. The effects of casein hydrolysate and 2,4-D on somatic embryogenesis and regeneration were examined. The callus induction procedure used mature seeds, which were manually dehusked and sterilised before being chosen as the explant source.

2.5. Regeneration

Using sterile forceps, elongated plumules were separated from the seed scutellum after callogenesis. For the in vitro regeneration of many shoots, scutellum-derived calli were employed as an explant.The callus was moved to an adapted Murashige and Skoog (1962) medium consisting of 3% sucrose, 4.33g/L of MS salt, 100mg/L of myo-inositol, 2mg/L of glycine, and 0.5mg/L each of nicotinic acid, pyridoxin, and thymine, as well as plant growth regulators such kinetin and NAA. Phytagel 2.8g/L was used to solidify the medium. 40W white cool fluorescence tubes produced 2000 lux of light. The culture the chamber had a temperature of 25°C and a relative humidity of 65%. The photoperiod was kept at 14 hours. Every week, visual observations of culture were made. Using the data gathered as a foundation, vitro regeneration has been improved by.

2.6. Acclimatization of Regenerated Plants

Plantlets were induced from this callus on regeneration medium. When plantlets were gained length 2-3cm in petri plates, they were shifted to jars on Plant growth regulators (PGRs) added to MS basal medium (Murashige and Skoog, 1962) i.e. kinetin 3mg/L and NAA 1mg/L. When regenerated plantlets were developed well root and shoot system, they were moved to clay pots with soil & peat moss (1:3) for additional development and growth in order to adapt to the surroundings.

3. RESULTS AND DISCUSSION

3.1. Optimization of Seed Surface Sterilization

It is necessary to decontaminate explants before culturing in *in vitro* conditions. For this purpose, an efficient sterilization protocols are needed. In present study, 3 methods for seed surface sterilization were tested.

In Fig. 1.0. a comparative evaluation of seed surface sterilization and seed survival is given for three methods used. It was observed that treatment A resulted the most effective control of contamination (100% control) and seed germination was also maximum (98%). On the other hand, other two methods resulted relatively less sterilization and seed survival rate were also less. As method B resulted in 98.3% surface sterilization and 95% seed germination while method C resulted in 96% surface sterilization and 96% seed germination.

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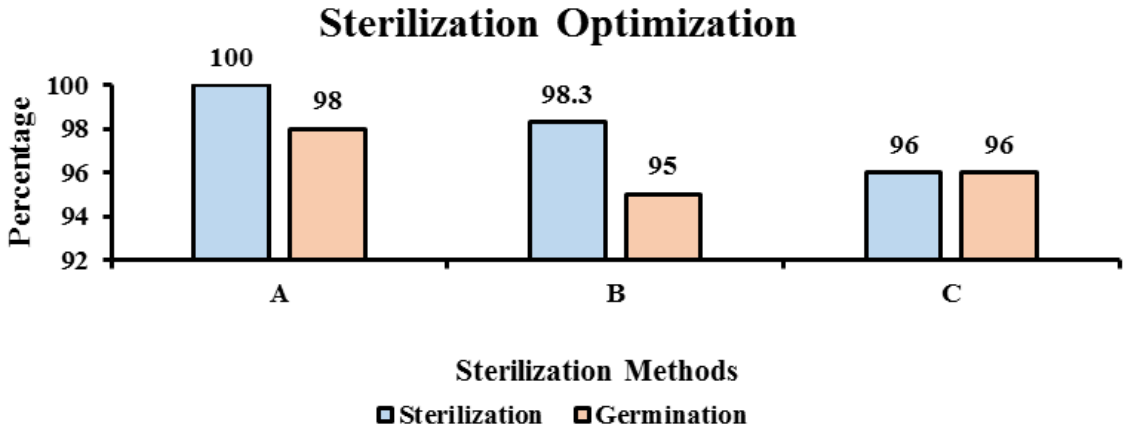

Fig. 1.0: Comparison of contamination control and seed survival percentage of three surface sterilization methods. **A)**. Methods A. Use of 0.1% $HgCl_2$ for 10 mints. **B).** Methods B. Use of ethanol (70%) for half minute and along with 0.1% $HgCl_2$ for 5 minutes only. **C)**. Methods C. Use of 70% Ethanol for half minute and commercial bleach (50%) for 25 min.

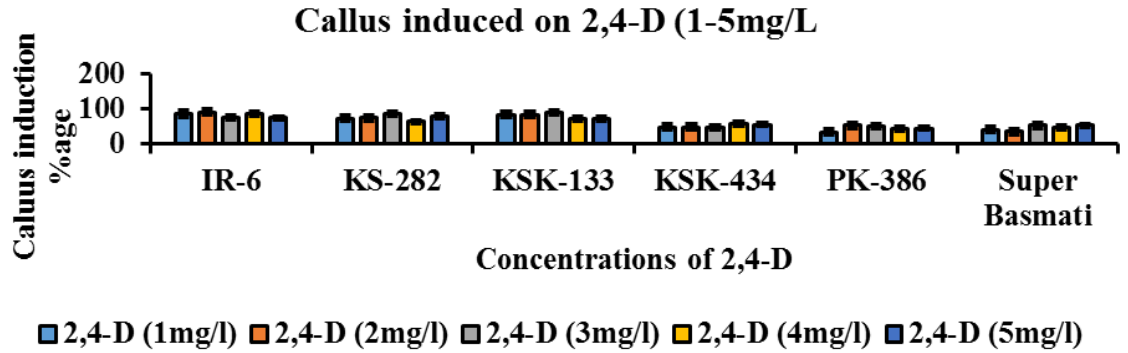

Fig. 2.0: Callus induction %age from mature seed derived scutellum explant of different varieties on MS media having 2,4-D (1, 2, 3, 4 and 5 mg/L) in them.

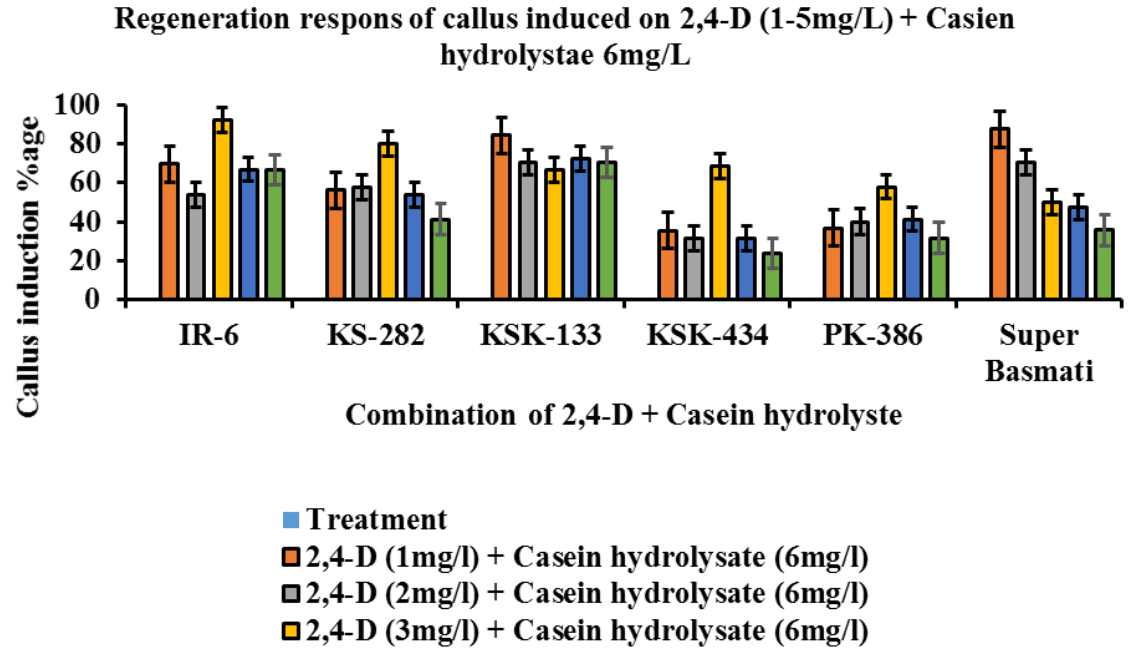

Fig. 3.0: Callus induction in different rice varieties on MS medium augmented with 6g/L casein hydrolyste and 2,4-D (1-5 mg/L).

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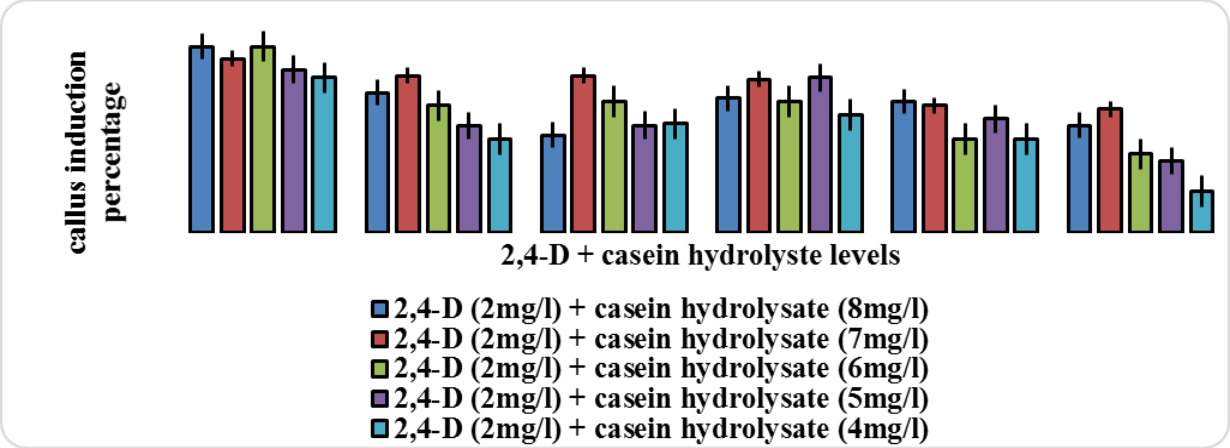

Fig. 4.0: Callus induction percentage on MS medium containing 2mg/L 2,4-D and (4-8mg/L) casein hydrolysate.

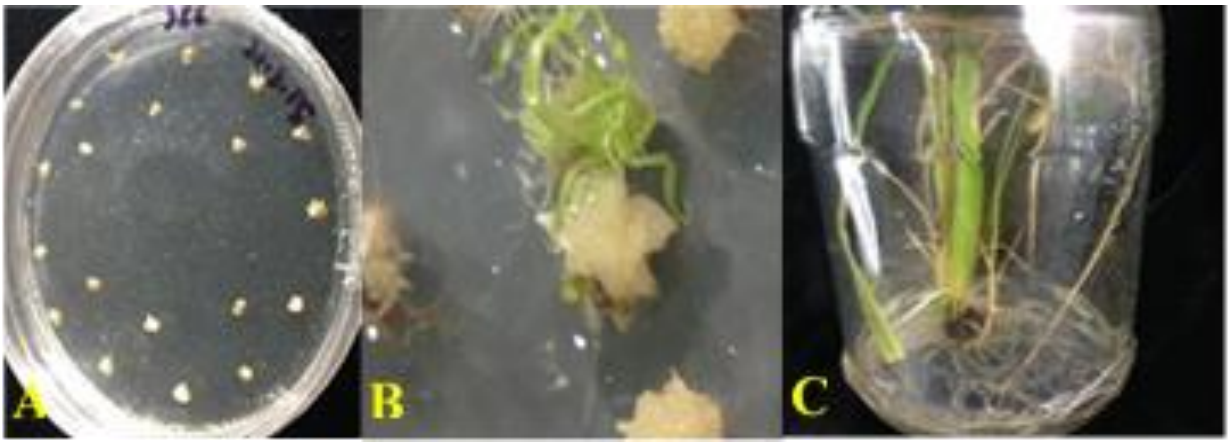

Fig. 5.0: Regeneration response of callus on MS medium supplemented with 1mg/L NAA and 3mg/L Kinetin.

3.2. Effect of 2,4-D Concentrations on Callus Induction

Mature seeds of six rice genotypes after surface sterilization using methods A, were cultured on MS media augmented with 5 different concentrations (1, 2, 3, 4 and 5 mg/L) of a synthetic auxin i.e. 2,4-D. After culture, the plates were incubated at 25±2 ºC under dark conditions for 5 days. After 5 days, callus was observed in all varieties with varying degrees of induction. Hence, it was concluded that callogenesis response genotype dependent. When different genotypes were compared, maximum callus induction %age was observed in IR-6 with 2,4-D @ 2mg/L while the minimum callus induction %age was found in Pk-386 with 2,4-D @ 4mg/L. Other varieties KS-282, KSK-133, KSK-434 and Super Basmati responded in between these two extremes.

3.3. Synergistic Impact of 2,4-D and 6mg/L Casein Hydrolysate for Callogenesis

In this research effort, it was found that an auxin i.e. 2,4-D when coupled with casein hydrolysate, their interaction poses a positive impact on callus induction and subsequent regeneration. Hence, we used a fix concentration (6mg/L) casein hydrolysate to evaluate its effect in combination with different doses of 2,4-D (1-5mg/L). Same, six rice genotypes were used to evaluate the usefulness of both these chemicals on callus induction and regeneration. Maximum callogenic response was obtained in IR-6 while the lowest was in Pk-386. Concentration of 2,4-D and casein hydrolyste was found to affect the process of callogenesis. In an earlier study, Shahsavari et al., (2010) reported that good callogenesis response was obtained when 600mg/L casein hydrolyste was used in combination with 2mg/L of 2,4-D.

3.4. Effect of Varying Concentrations of Casein Hydrolysate in Combination with 2,4-D

In present study, we obtained considerable callus induction with very low concentration of casein hydrolysate 6mg/L which was much lower than the reported ones. In a further experiment, we tried to optimize the concentration of casein hydrolysate with more callus induction response. Hence, callus induction response was

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determined at 2mg/L of 2,4-D (fixed) in combination with casein hydrolyste (4-8 mg/L). Maximum callus induction was observed with at 2mg/L of 2,4-D and at 4mg/L of casein hydrolystae. As far as genotypic response is concerned, the highest callus induction was obtained in IR-6 and the lowest in Pk-386.Other varieties responded in between. A previous study by Khaleda (2006) reported that callus induction percentage increased by addition of casein hydrolysate and highest callus induction percentage was observed on MS media having 2,4-D 2mg/L and 0.6% (w/v) casein hydrolysate.

3.5. *In vitro* Regeneration

Efficient *in vitro* regeneration in monocotyledonous plants is of pivotal importance for various biotechnological applications including genetic engineering as well as genome editing (Oladosu et al., 2019). In present study, calli were detached from seeds and cultured on already reported regeneration medium (Joyia and Khan, 2012). Regeneration of calli was found to be highly genotype dependent. In some previous study (Joyia and Khan, 2013), it was also observed to be linked with the age of calli and the concentration of plant growth regulator.

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