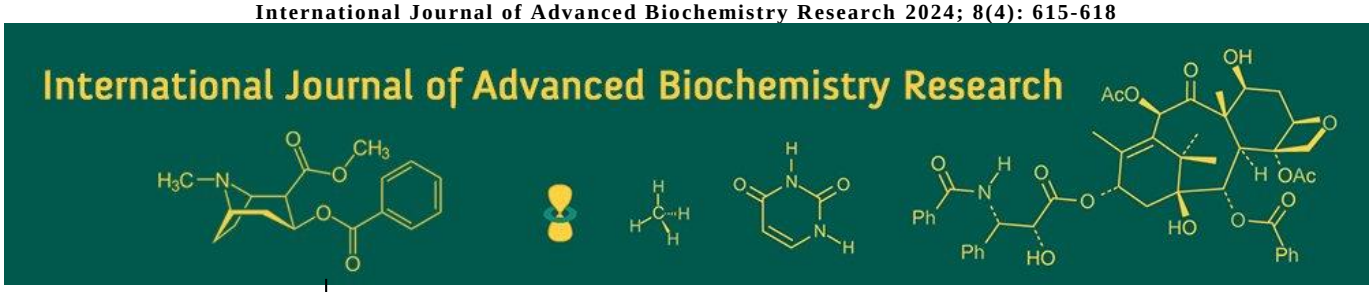

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Optimization of callus induction under different growth regulators in sugarcane (*Saccharum officinarum* L.)

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Abstract
Two sugarcane cultivars (COLK 14201 and CO 15023) were used to induce callus, and the response of various Plant Growth Regulators (PGR) to this process was assessed. Different concentrations were selected for the best callus induction after optimization. The combination of 2,4-D alone and in combination with the other growth regulators (BAP and kinetin) was used for callus culture. The best callus response in the terms of number of days, callus fresh weight and callus frequency in COLK 14201 was observed on MS media provided with 2,4-D (4 mg L^{-1}) while in CO 15023 the best response was on MS + 2,4-D (3 mg L^{-1}) + Kinetin (0.5 mg L^{-1}). The maximum callus frequency was observed 100 percent in 2,4-D (3 mg L^{-1}) but callus was not good than 2,4-D (4 mg L^{-1}). Other parameters were also recorded like callus texture and callus color.

Keywords: Callus, COLK 14201, CO 15023, 2,4-D, BAP, Kinetin

Introduction
Sugarcane (*Saccharum officinarum* L.) is a major agricultural cash crop grown in nations with subtropical and tropical climates, including the United States, India, Pakistan, China, Brazil, Australia, and Cuba. Sugarcane is an industrial crop primarily used to manufacture sugar and bioethanol. It produces sustainable bioenergy and a variety of other byproducts (Gomathi *et al.*, 2020) [5]. It is a significant industrial crop that provides 70% of the sugar consumed worldwide, making it a main source of sugar. It is a big grass belonging to the Poaceae family and genus Saccharum (Cursi *et al.*, 2022) [3]. According to estimates from the US Department of Agriculture (USDA), 1.87 billion tons of sugarcane were produced worldwide in crop year 2021-22 on an area harvested to the tune of about 26.4 million hectares. Brazil is one of the world's top producers of sugarcane and a major global source of both sugar and ethanol. The nation harvested 5.15 million hectares of sugarcane in 2021-2022, yielding 431.81 million tonnes of sugarcane While Uttar Pradesh is the greatest sugarcane growing state in India, with an area of 2.18 million hectares and production of 177.43 million tonnes (Directorate of Economics and Statistics, DA&FW, 2021-22). The resulting types are polyploids and aneuploids with chromosome counts ranging from 80 to 120 (Hussin *et al.*, 2022) [7]. The ideal temperature for sprouting (germination) of stem cuttings is 32 °C to 38 °C. Average daily temperatures between 22 °C and 30 °C promote optimal growth. The minimal temperature for active growth is around 20 °C. Lower temperatures (20 °C to 10 °C) are ideal for sugarcane ripening, as they reduce vegetative growth rate and increase sucrose content. Sugarcane juice is a refreshing drink that contains vitamins, carbs, and amino acids. In the sugar business, approximately 493 megagrams of bagasse were recovered from around 1,600 Mg of sugarcane (Khattab *et al.*, 2019) [10]. In order to address these various issues and attain a substantial output of sugarcane, scientists are concentrating on biotechnological methods like micropropagation (Dinesh *et al.*, 2015) [4]. Large-scale production as well as the development of disease-resistant and disease-free plants are made possible by this technique. Due to its many benefits, this method has found commercial use all over the world (Salokhe *et al.*, 2021) [18]. Because each genotype's reaction varies depending on the kind and dosage of the plant growth regulator, each stage of micropropagation requires an effective methodology (Lal *et al.*, 2015) [11].

To achieve successful micropropagation, it is therefore essential to determine which plant growth regulator or combination of regulators will result in a high rate of growth in the shortest length of time (Hailu *et al.,* 2018) [6]. In plant tissue culture, the most commonly utilized Plant Growth Regulators (PGRs) for this purpose are cytokinin and auxin. The kind of established culture and the plant cells' formative process are determined by the ratio of auxin to cytokinin (Purnamaningsih *et al.,* 2021) [16]. The majority of micropropagation studies focus on the impact of cytokinin and auxin, both alone and in combination, on callus induction (Subedi *et al.,* 2015) [20]. Different combinations of PGR were studied for callus induction of two sugarcane (*Saccharum officinarum* L.) genotypes (COLK 14201 and CO 15023) with the aim of providing assistance with the development of a protocol and assessing the different roles of plant growth regulators. The present study highlights the significance and uniqueness of micropropagation technology for each genotype in sugarcane.

Materials and Methods

Collection of Mother Plant

For the study, the Germplasm of two sugarcane genotypes (COLK 14201 and CO 15023) were collected from UPCSR Muzaffarnagar (UP). The experiment was conducted in the Tissue Culture Lab, Division of Plant Biotechnology, College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut (UP) India.

Explant Sterilization

Plant material was first washed through the running tap water for 20 to 30 minutes and use 0.2% (w/v) Bavistin for 10 minutes, washed with sterile distilled water for 5 minutes and kept in autoclaved jars then transfer to laminar airflow cabinet. The young meristem explants were treated with 70% alcohol for 30 second to 1 minute, followed by another treatment in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. Finally, the young meristem cuttings were washed thoroughly 3 to 5 times with sterile distilled water before the inoculation in to sterilized nutrient agar media pre-packed in culture tubes. All the above operations were performed under aseptic conditions in laminar airflow cabinet.

Media Preparation and Pouring

Glassware was autoclaved for 15 to 20 minutes at 121 °C and 15 pressure to sterilize it. For the purpose of explant cultivation, Murasige and Skoog Media was used Ahloowalia and Maretzki, (1983) [2]. The pH value of MS media was adjusted to pH 5.7-5.8 by adding HCl (0.1 M) or NaOH (0.1 M). The test tube was filled with 20 ml culture media, and a 25 mm seal was placed on top. It had previously been autoclaved to achieve sterilization and covered with polypropylene sheets.

Inoculation Process

The innermost layer of apical meristem was selected from the 3 to 4 month old sugarcane plant (Saleem *et al.,* 2022) [17] as the explant culture on the MS media surface. The explant was inoculated on the surface of MS medium. To provide an aseptic environment, 70% ethanol was used to clean the inoculation area and instruments also. Before explant inoculation, the UV rays of laminar airflow was turned on for 20 minutes. The innermost layer of apical meristem that cut into a small discs was 0.5 mm thick (Saleem *et al.,* 2022) [17] and then discs was inoculated on MS media. Test tube were sealed with parafilm and incubated at 25 ± 2 °C in the dark condition.

Callus Induction

In order to record callus induction, various plant growth hormones were used in conjunction with the composition of MS media. After 30 days, callus frequency, callus weight, and number of days required to induce callus were recorded. The explant selection was from the innermost layer of apical meristems before being placed on MS media with different concentrations of 2,4-D alone and with other plant growth regulators (Srivong *et al.,* 2015) [19].

Table 1: Different compositions of plant growth regulators for callus induction

Treatments	Composition
T_1	MS + 2,4-D (3 mg L^{-1})
T_2	MS + 2,4-D (4 mg L^{-1})
T_3	MS + 2,4-D (3 mg L^{-1}) + BAP (0.5 mg L^{-1})
T_4	MS + 2,4-D (4 mg L^{-1}) + BAP (0.5 mg L^{-1})
T_5	MS + 2,4-D (3 mg L^{-1}) + Kinetin (0.5 mg L^{-1})
T_6	MS + 2,4-D (4 mg L^{-1}) + Kinetin (0.5 mg L^{-1})

Results and Discussion

Callus Induction

All the treatments significantly affected the callus induction, while genotypes and the effect of the interaction between treatments and genotypes were also significant.

For COLK14201, Explant kept on MS Media with different hormonal concentrations. The different frequencies of callus were recorded from each and every treatment according to increasing as well as decreasing the concentration of 2,4-D with BAP and Kinetin showed in fig.1. and other parameters were also recorded like callus induction, callus weight, callus color and callus texture. The best callus selected in MS + 2,4-D (4 mg L^{-1}) (T_2) on the basis of callus weight and texture with 95% frequency where callus induction of 2, 4-D (4 mg L^{-1}) was started after 9 days of explant inoculation. The optimization of 2,4-D with kinetin was better than 2,4-D with BAP. Therefore among 9 tested combinations of media of each treatment and average weight of callus after 30 day was recorded more than CO15023. The different stages of callus formation after 10, 20 and 30 days can be seen in fig.1. The callus of COLK 14021 was shown fig.1 in the form of light yellow, granular and friable.

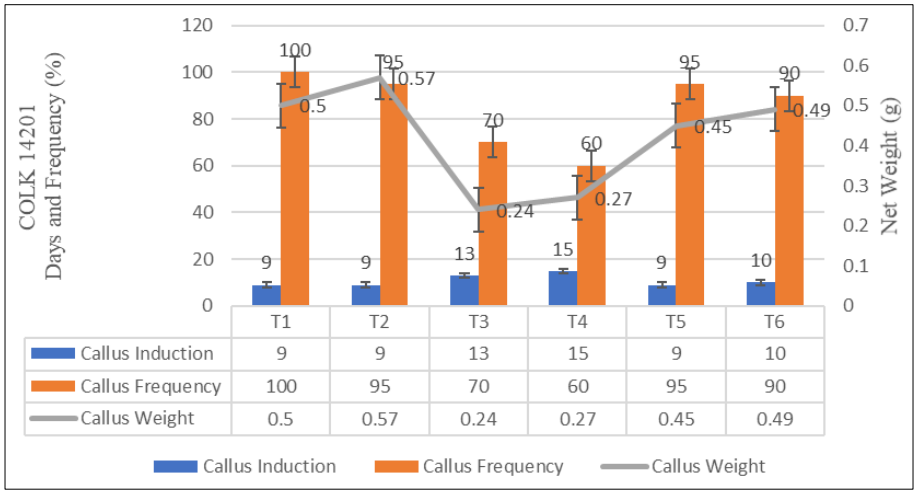

Fig 1: Effect of growth regulators on callus induction in term of callus, number of days and frequency in COLK 14201

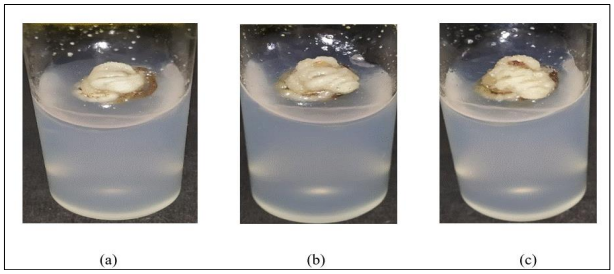

Fig 2: Callus of COLK 14201 on MS + 2,4-D (4 mg L^{-1}) after 10, 20 and 30 days of inoculation (2X)

In CO 15023, Different callus frequencies were recorded from among various treatments under aseptic environments; however, callus weight and highest frequency were shown in this concentration MS+2,4-D (3 mg L^{-1}) + kinetin (0.5 mg L^{-1}) (T_5) than BAP. The maximum callus weight of CO 15023 was found with 2,4-D (3 mg L^{-1}) + kinetin (0.5 mg L^{-1}) (T_5), whereas less callus weight was found in 2,4-D (3 mg L^{-1}) than (T_5). The highest frequency of (T_5) was recorded at 30 days after explant inoculation. Different stages of callus formation after 10, 20 and 30 days can be seen in Fig. 3. The callus of CO 15023 was creamy, granular and friable.

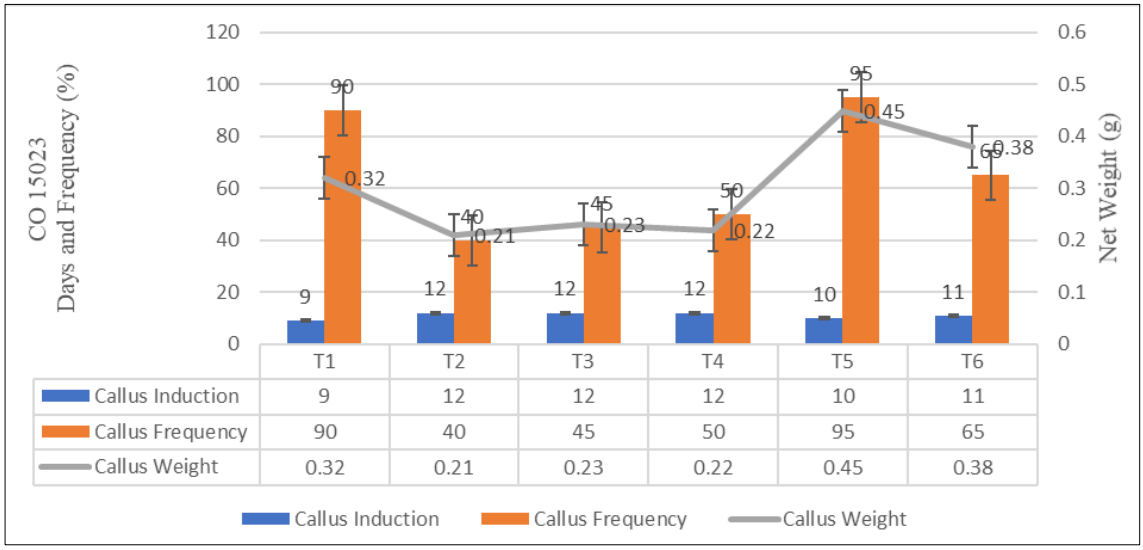

Fig 3: Effect of growth regulators on callus induction in term of callus, number of days and frequency in CO 15023

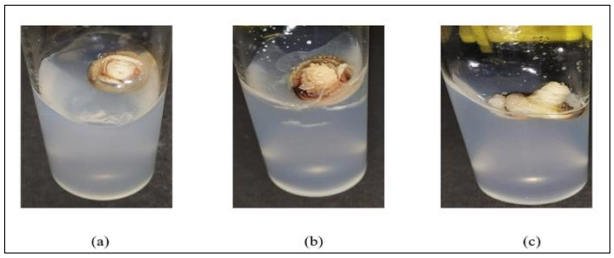

Fig 4: Callus of CO 15023 on MS + 2,4 D (3 mg L^{-1}) + Kinetin (0.5 mg L^{-1}) after 10, 20 and 30 days of inoculation (2X)

Discussion

Callus induction in sugarcane is influenced by various factors and genotypic interaction with plant growth regulators, with the endogenous level of hormones reflecting the callus response (Phamontree *et al.*, 2022) [15]. The optimization of callus growth under different hormonal concentrations by which we were select which one hormonal concentration is good for callus growth and development (Kaur *et al.*, 2018) [9]. Callus induction is mostly carried out using different concentrations of 2,4-D alone and along with BAP and kinetin combination also. Meanwhile, auxin and cytokinin are known to accelerate callus growth and development (Saleem *et al.*, 2022) [17]. Kinetin alone was unable to induce the cytoplasm of parenchyma cells and remained highly vacuolated. 2,4-D is required for callus induction, whereas 2, 4-D and Kinetin promotes its proliferation. Thus, it is obvious

that auxin induced the callus, while Kinetin helped with proliferation (Mastuti *et al.*, 2017) [14]. The present study indicates that callus induction and proliferation were also achieved at 2,4-D (3 mg L^{-1}), and higher concentrations of 2,4-D (4 mg L^{-1}), its callus induction potency was positively affected in the COLK 14201 genotypes but not in CO 15023. In the present study, callus induction and proliferation decreased when the concentration of 2,4-D (3 mg L^{-1}) + BAP (0.5 mg L^{-1}) and 2,4-D (4 mg L^{-1}) + BAP (0.5 mg L^{-1}) taken (Iqbal *et al.*, 2016; Tripathy *et al.*, 2020) [8, 21]. The highest callus mass being found in 2,4-D (3 mg L^{-1}) but quality was not good compare than 2, 4-D (4 mg L^{-1}) in COLK 14201 and highest mass of callus were shown 2,4 D (3 mg L^{-1}) + Kinetin (0.5 mg L^{-1}) in CO 15023. The callus surface exhibited compact and nodular features, indicating an embryogenic callus (Srivong *et al.*, 2015) [19] reported similar observations.

Conclusion

From the above results, it was concluded that callus induction of two genotypes of sugarcane was optimized using different growth regulator and find out good callus quality on selective hormonal concentration that was mention in above. This study also highlights the importance of 2, 4-D and 2, 4-D with Kinetin for callus induction. Eventually, a complete optimize protocol will be able to be used in commercial laboratories to shooting and rooting then propagate sugarcane plant or conduct any experiments on the above genotypes.

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