Eco. Env. & Cons. 30 (July Suppl. Issue) : 2024; pp. (S242-S246)
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ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2024.v30i04s.043

Scanning Electron Microscopy Investigation of Cytoplasmic Male Sterile Line Rice : Exploring Treatment Effects on Plant Components and their Impact on Hybrid Rice Seed Production

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(Received 8 November, 2023; Accepted 13 January, 2024)

ABSTRACT

In addressing the challenge of inflorescence emergence in cytoplasmic male sterile line (CMS) rice, specifically the CMS A-line, GA_3 has conventionally been utilized. However, the high cost of GA_3 poses financial constraints for middle-class and marginal farmers. This study aims to identify cost-effective alternatives to GA_3 for CMS breeding. Utilizing IR58025A as the A-line and KMR3 as the R-line, obtained from Rice Research Station, Chinsurah, Hooghly, the experiment was conducted following CMS breeding layout design at the Crop Research Farm under the Department of Botany, the University of Burdwan. Various chemicals, including GA_3, penicillin, sulfonamide, and gentamicin, were applied as foliar spray treatments, alongside a control group, during flowering to produce F_1 seeds from the A-line in the boro season. The primary objectives include exploring microfeatures of different plant organs such as leaves, stems, androecium, and gynoecium through SEM analysis to evaluate the effectiveness of the diverse treatments. This research contributes valuable insights for rice breeders and the scientific community, offering potential alternatives to GA_3 in CMS breeding technology.

Key words: *CMS, Penicillin, F_1 seeds, SEM*

Introduction

The go-to method for maximizing hybrid rice technology commercially is the cytoplasmic male sterility (CMS) system, commonly referred to as the three-line system. This system involves the interplay of a CMS line, a maintainer line (B line) sharing the same nucleus as the CMS line (A line), and a restorer line (R line). Cytoplasmic male sterility (CMS), a maternally inherited trait, prevents the production of functional pollen grains and has been observed in more than 150 plant species (Fujii and Toriyama 2009). According to El Shamey *et al.* (2022) cytoplasmic male sterility (CMS) provides an irreplaceable strategy for commercial exploitation of heterosis and producing high-yielding hybrid rice. The exogenous application of plant growth regulators could improve outcrossing rates of the CMS lines by affecting floral traits and accordingly increase hybrid rice seed production. The utilization of gibberellic acid (GA_3) in rice breeding poses a financial challenge for middle-class and marginal farmers due to its high

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cost. To address this concern and facilitate broader access, it would be advantageous for rice breeders and the scientific community to investigate alternative, cost-effective chemicals that can serve the same purpose in this breeding technology (Pal *et al.,* 2023). This exploration aims to identify viable substitutes for GA_3, making rice breeding more economically accessible to a wider spectrum of farmers. Keeping all these views in mind IR58025A as A-line, and KMR-3 as R-line were procured from Rice Research Station, Chinsurah, Hooghly which were grown in the research plot in the Crop Research Farm under the Department of Botany, the University of Burdwan following the norms of CMS breeding layout design. Scanning electron microscopic(SEM) study of epidermal features of rice leaf epidermis of BRRI DHAN 29 was done by Islam *et al.* in 2009 and morphological-histological investigation on functional male sterility of cultivated rice (*Oryza sativa* L.) was done by Cheng and Huang *et al.* in 1980. The main aims and objectives of this experiment was to study the microfeatures of different plant organs like leaf, stem, androecium and gynoecium etc.of IR58025A (CMS line rice) for the effectiveness of different treatments compairing with control set by SEM study.

Materials and Methods

IR58025A (CMS line) and KMR3 (R-line) were procured from Rice Research Station, Chinsurah, Hoogly which were grown in the research plot in the crop research farm under the Department of Botany, the University of Burdwan in boro season. The experiment was laid out in a randomized block design method with three replications during boro season of 2011-12. R line and A line were transplanted in the ratio of 2:6. Five treatments including control were applied as foliar spray on A line plants for F_1 hybrid rice seed production. Treatments and doses of chemicals are exhibited in Table 1.

The following protocol was adopted for the purpose of SEM study by using S530 HITACHI, scanning electron microscope, Japan.

For histological and scanning electron microscopic (SEM) observation, the leaves and the spikelets of CMS plants (IR58025A) were collected from experimental field treatment-wise. For the preparation of biological specimen of SEMstudy, the first step was adopted like 'fixation'. The materials were put into 2.5% gluteraldehyde for 2 hours for fixation. The next step was to dehydrate the tissue by passing it through a series of alcohol solution. This step termed as 'dehydration' which was done by using ethanol. After 2 hours the materials were shifted to 50% alcohol for 5 minutes and then 70% alcohol for 30 minutes in 2 changes and then 90% alcohol for 30 minutes in 2 changes. After that the materials were passing into the absolute alcohol for 30 minutes in 2 changes and then the materials were immersed in absolute alcohol : amyl acetate in the ratio of 3:1, 2:2 and 1:3 for 30 minutes in each case. Besides the materials were preserved in amyl acetate for 30 minutes and the preserved leaves were kept for 2 days. After 2 days, leaves were cut into small pieces for CPD. As liquid CO_2(critical temperature 31.1^0 C) is suitable due to low critical point temperature, we set the same and kept the settings for 5 minutes and opened the leakage valve and pressure slowly goes down as zero and after that, the specimen released from the chambers of cassette by opening the cap of further procedure.

After CPD, specimens were mounted with adhesive to a metal stab. The mounted specimen was coated with a thin layer (20 to 30 nm.) of a conductive metal. The metal coating (gold coating by IB2 ion coater) provides more escape volume of electron and prevents the breakage of fine structure due to electron bombardment.

After metal coating the stab with surface details of the experimental specimen were observed under the electron microscope using accelerating voltage of 15 or 20kv.on the secondary electron emission mode.

Four metrical characters were also studied till harvest: i) Panicle exertion length (cm) ii) Total no. of grain panicle $^{-1}$ iii) Grain yield plant^{-1}(g) and iv) 1000 grain weight(g).

Results and Discussion

Various morphological characters were studied till harvesting of the crop and mean values were ob-

Table 1. Treatments and Doses of chemicals

Treatments	Chemicals applied	Doses (ppm)
T_1	Control	-
T_2	Penicillin	400
T_3	Sulfonamide	100
T_4	Gentamycin	100
T_5	GA_3	60

tained which are shown below in Table 2.

It was critically observed various peculiarities during cropping time till harvesting. The length of panicle exsertion was found to be highest (21.27cm) in treatment- 2 (Penicillin) and lowest length (16.93 cm) of exserted panicle in case of treatment-1 (control). Grain yield plant-1 also shows similar result i.e. highest (69.24 g) in treatment -2 (penicillin) and lowest (64.13 g) in case of treatment-1(control). Accordingly the exserted panicle length was observed 20.20 cm and 21.03 cm in case of gentamycin and GA_3 respectively. In other major yield characters *viz.* total no. of grains panicle^{-1}, grain yield plant^{-1}(g) and 1000 grain weight (g) were also studied.

The observed data reveals notable trends in the impact of various treatments on the A line rice plant during the Boro season (2011-12). Treatment-2 consistently exhibited the highest recorded values across all cases, while treatment-3 consistently showed the lowest performance. Notably, treatments 4 and 5, involving gentamycin and GA_3, demonstrated a parallel sequence akin to panicle exsertion length. Treatment-1, serving as the control set (T_1), consistently exhibited the lowest performance across all metrical characters, affirming its effectiveness as a baseline reference.

The inclusion of a scanning electron microscopic (SEM) study provided valuable insights into the morphological alterations induced by different treatments on the male sterile A line rice plant (IR58025A). The SEM analysis, as illustrated in Fig.1 (a)-(o), clearly delineates variations in different plant organs corresponding to distinct treatments.

In Fig.1, scanning electron micrographs (SEM) images showed that the rice leaf surface comprises of long and short cells, stomata with guard cells, macro hairs, prickle hairs, papillae and silica bodies. In this experiment a special type of macro hair was present in Fig. 1.a. This macro hair is small and slender in shape with blunt tip. In rice, silica body content might be correlated with fungal diseases and insect pests (Kim *et al*., 2002, Prychid*et al*., 2003, Lux *et al*., 1999, 2003). Here, triangular silica bodies are clearly visible in respective slides (Fig 1.a, d, g, j and m). It has been speculated that silica structures provide mechanical support for the leaf (Yamanaka *et al*., 2009). The number of silica bodies and rows per ridge might be diagnostic for the specific treatment. But there was always a range of variations due to the age of seeds and the environmental conditions must be taken into account (Kim *et al*., 2002; Prychid *et al*., 2003).

Here ladder like structures are clearly visible in leaf structures (Fig.1.a, d, g, j, m). According to Yamanaka *et al*. (2009) the ladder like structures inhibit the flat leaf from undergoing twisting torsions which may assist the leaf to absorb sun-light more effectively. It should also be mentioned that the flexural rigidity perpendicular to the plane of the leaf was found to increase with increasing silica content. In control set, the endothecium layer was compact in (Fig. 1.b) but in case of GA_3 and penicillin treatment, features exhibited the loosening of endothecium layers (Fig. 1.e, h). Foliar application of GA_3 at the heading stage enhances floret opening duration, stigma properties, panicle exertion of male sterile lines, rate of stigma exertion, out-crossing rate and seed yield potential during hybrid rice seed production, as reported by Wang *et al*. (2019). Mukherjee and Wareings (1983), also stated that the levels of gibberellin and cytokinin like substance are increased in mungbean seedlings by penicillin treatment.

The most significant difference in indehiscent anthers from normal ones is their strongly thickened fibrous endothecium (Cheng and Huang *et al*., 1980). This might be the indicator of the active nature of function for disseminating the pollens throughout the pollination period. The arrow-shaped anther lobe of penicillin treated plant was very prominent and attached with the filament tightly. It was looking healthy (Fig.1k). Obviously it was more elongated and widened than that of the control set (Fig.

Table 2. Data showing mean values of various metrical characters at a glance

Season	Treatment	Panicle exsertion length (cm)	Total no. of grain panicle^{-1}	Grain yield plant^{-1} (g)	1000 grain weight (g)
Boro (2011-12)	T_1	16.93	189.36	64.13	18.23
	T_2	21.27	206.87	69.24	20.54
	T_3	19.93	201.54	66.18	18.54
	T_4	20.20	202.24	65.66	20.64
	T_5	21.03	203.29	67.36	19.13

1b). The gentamycin and sulphonamide treated anther lobes (Fig. 1h) and (Fig. 1n) are not so prominent like penicillin treated anther lobe (Fig.1.k). The gynoecium was found to be more fibrous, healthy, prickle hairs with more gametophicant than that of control set (Ramaer, 1935, Schmid, 1976). Fig.1 shows that the penicillin treated gynoecium was also fibrous and healthy than that of control set (Fig.1c) and other treatments applied. Sulphonamide treated gynoecium (Fig.1.o) is less fibrous than penicillin treated gynoecium but healthier than control set. As a result pollination might occur more and more than that of control set whereas, F_1's of the crosses should be a wide range of seed set percentage. The sterility was dependent with the difficulty in anther dehiscence, flower synchronization and other environmental factors like humidity, wind direction and speed, atmospheric temperature etc. (Werbster and Sing, 1964).

Conclusion

In the course of this study, penicillin emerged as the

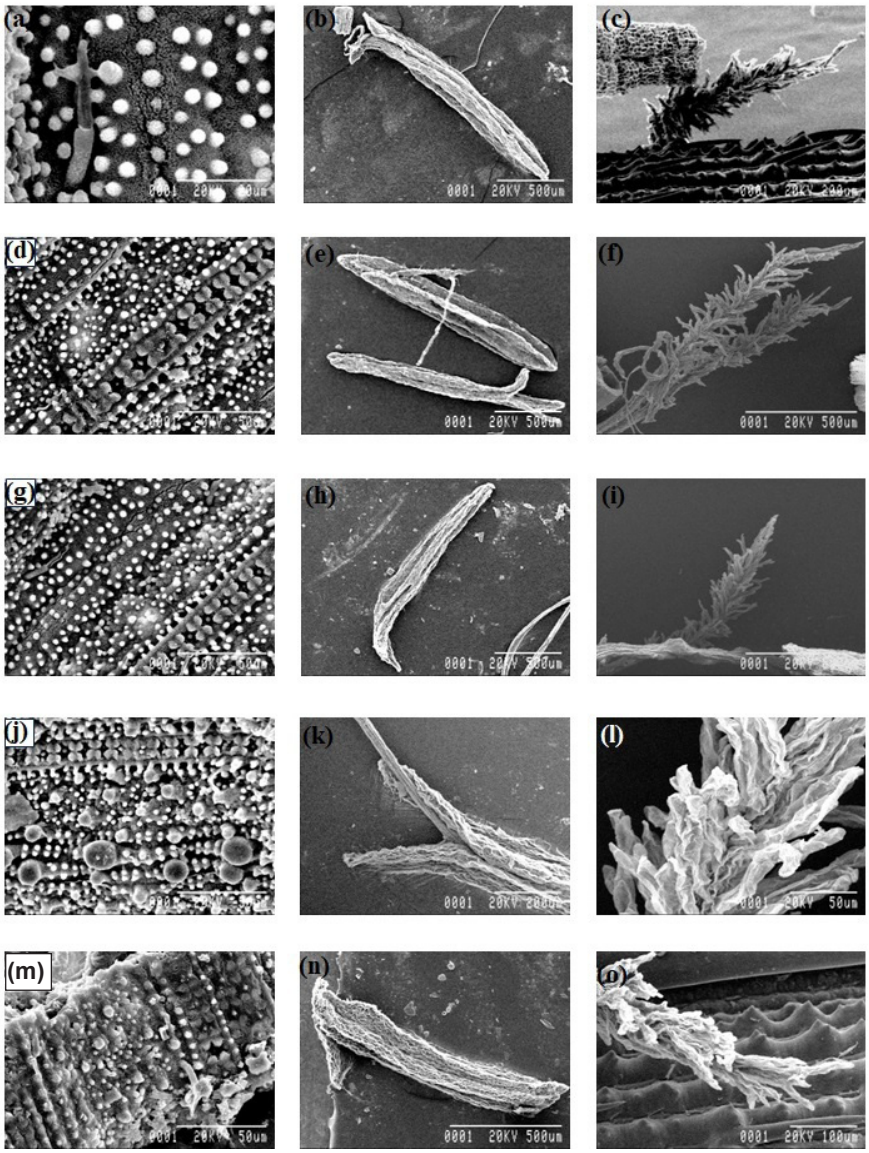

Fig. 1. Scanning electron micrograph (SEM) images of (a) leaf at control, (b) stamenat control and (c) gynoecium at control. GA_3 treated (d) leaf, (e) stamen and (f) gynoecium respectively. Gentamycin treated (g) leaf, (h) stamen and (i) gynoecium respectively. Penicillin treated (j) leaf, (k) stamen and (l) gynoecium respectively. Sulphonamide treated (m) leaf, (n) stamen and (o) gynoecium respectively.

most promising treatment, demonstrating optimal results for panicle emergence at a comparatively lower cost, while simultaneously yielding higher crop output. Following closely, GA_3 and gentamycin exhibited commendable performances in terms of the treatment efficacy.

The micro-histological scrutiny of various plant organs in the CMS line rice (IR58025A) under diverse treatments, as conducted through SEM, provides crucial insights. Specifically, penicillin treatment induced a notable loosening of the endothecium layer in the androecium, coupled with the development of a more fibrous, robust, and gametophicant gynoecium. These microstructural enhancements signify a heightened potential for seed set percentage in F_1 hybrid rice production. Such detailed observations not only augment our understanding of the underlying physiological processes but also hold implications for refining breeding strategies and optimizing agronomic practices in rice cultivation.

This comprehensive investigation not only highlights the practicality of penicillin as a cost-effective alternative for optimizing panicle emergence and yield but also underscores its potential for positively influencing reproductive structures critical for successful F_1 hybrid rice seed production. The implications of these findings extend to the realm of crop improvement strategies, offering valuable guidance for the agricultural community seeking sustainable and economically viable approaches in hybrid rice seed production.

Acknowledgements

The authors are grateful to the authority of the University of Burdwan and Rice Research Station, Chinsurah, Hoogly for necessary help. Authors also express deep sense of gratitude to Dr. Jagatpati Tah, for providing valuable guidance as and when needed for this study.

Conflict of Interest

The authors declare that no conflict of interest exists.

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