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RESEARCH ARTICLE

Influential factors in haploid embryo induction of triticale (× *Triticosecale*) through wide hybridization with maize (*Zea mays* L.)

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Abstract

Generating doubled haploids (DHs) is crucial for accelerating the breeding process and facilitating the creation of crop-mapping populations. Although other cultures or pollination techniques with *Hordeum bulbosum* have proven effective for haploid production in common wheat, similar efforts in triticale have met with limited success. Cross breeding with maize was employed to generate haploid seedlings and subsequently, fertile DHs in triticale. The current research investigates the effect of four different auxin treatments for post-pollination application in triticale × maize crosses, using combinations of 2, 4-D (2,4-dichlorophenoxyacetic acid), silver nitrate and dicamba. Among the four treatments, T_3 (2, 4-D + dicamba at 100 mg/L + 85 mg/L, respectively) and T_4 (2, 4-D + dicamba at 100 mg/L + 100 mg/L, respectively) were the most effective in inducing haploid embryos and achieving plant regeneration. The frequencies of haploid embryo induction were 31.46% and 30.61%, while plant regeneration frequencies were 11.53% and 11.11%, respectively. Determination of the phytohormone combination and its concentration is vital to affecting haploid embryo induction in triticale (× *Triticosecale*). Following wide hybridization with maize (*Zea mays* L.) has opened new possibilities in the triticale breeding program.

Keywords: haploid; maize; phytohormones; triticale; wide hybridization

Introduction

Triticale is the first man-made cereal crop that is self-pollinated and made from wheat (*Triticum*) and rye (*Secale*) (1). Triticale often combines rye's resistance to disease and environmental factors, particularly soil conditions, with wheat's high yield potential and superior grain quality. As a result, triticale is a crop that thrives in marginal conditions (soils that are prone to acidity or dryness) or heavy disease pressure. Triticale can resemble either of its parents to varying degrees, depending on the cultivar. It has drawn interest recently as a possible energy crop and studies are now being done on the crop's biomass's potential for producing bioethanol. Triticale's potential applications as a grain and as a pasture crop have sparked interest in the crop.

However, the use of triticale relies on breeding initiatives, which yield novel cultivars possessing enhanced utility attributes or superior adaptation to environmental conditions (2). Enhancing genetic diversity in crops will strengthen their resilience against biotic and abiotic stresses, which will continue to be significant agricultural challenges (3). Achieving homozygosity using traditional breeding methods takes a long time and is tedious. Using this breeding technique, a single cultivar may take up to 14 years to develop. Combining biotechnological methods with traditional plant breeding practices can expedite breeding programs (4). Around the world, haploid bread and durum wheat are successfully produced by the chromosomal elimination process, which naturally takes place during wide hybridization in wheat (5). For bread wheat, haploid embryo induction (HEI) is essential to crop improvement programs. Both egg cells (female gametes) and microspores (male spores), which are haploid cells in the gametic developmental pathway, are the sources of double haploid (DH) plants. Numerous uses for DH plants can be found in genetics, plant breeding and basic biological research (6).

Double haploid breeding reduces the time required to achieve complete homozygosity while increasing selection efficiency in crop breeding (7). This approach is an alternative to different traditional breeding methods (8). Various techniques are available for generating haploid wheat plants, such as microspore or anther cultures, megaspore or ovule cultures and wide hybridization. Wide hybridization involves crossing of different species within the same genus (interspecific cross) or between different genera within the same family (intergeneric cross); for example, wheat × rye, *Oryza sativa* × *Oryza perennis*. Triticale, a hybrid between wheat and rye, was first

observed to be sterile by Wilson in 1875, but it was later developed into a fertile hybrid (9). In triticale × maize, inter-specific cross-embryo formation takes place without the presence of endosperm (10). When crossed with maize, distantly related cereals are practised by applying 2, 4-D (11, 12). The use of the plant growth hormone, 2,4-D, enhances seed growth in wheat and maize crosses. This hormone can be administered through spraying or droplet, applied to the florets or injected immediately or on the day after pollination (12).

Therefore, based on the essential factors mentioned above, the current study was designed to identify and evaluate embryo production efficiency with different genotypes.

Materials and Methods

Six lines of triticale (× *Triticosecale*) (IC642661, IC642662, IC642723, EC490146, EC490149, EC490148) used as the female was provided by National Bureau of Plant Genetic Resources (NBPGR), New Delhi and maize (*Zea mays* L.) genotype (PMH 10) used as the male was collected from a regional seed distributor in Jalandhar, Punjab.

The emasculated triticale spike was pollinated with freshly collected maize pollen for 15-20 min.

Four different treatments were applied in the form of injections as follows: T_1: 2, 4-D + $AgNO_3$ (silver nitrate) at 100 mg/L + 60 mg/L, respectively; T_2: 2, 4-D + $AgNO_3$ at 100 mg/L + 80 mg/L, respectively; T_3: 2, 4-D + dicamba at 100 mg/L + 85 mg/L, respectively; T_4: 2,4-D + dicamba at 100 mg/L + 100 mg/L, respectively, (without any control group) were injected to the uppermost internode of pollinated triticale spikelet at 24, 48 and 72 h of pollination. The spikes were collected from the plant base after 14-18 days since pollination (Fig. 1). Haploid plants were rescued after 16-20 days after pollination when the immature seeds turned translucent (13).

Seeds were washed with 70% ethanol to prevent contamination (Fig. 2). Seeds were sterilized with 0.2% mercuric chloride ($HgCl_2$) and 0.1% bavistin under a laminar air flow for 2-5 min. All the pseudo seeds were dissected

Fig. 1. Pollinated spikes are harvested 14-18 days after pollination.

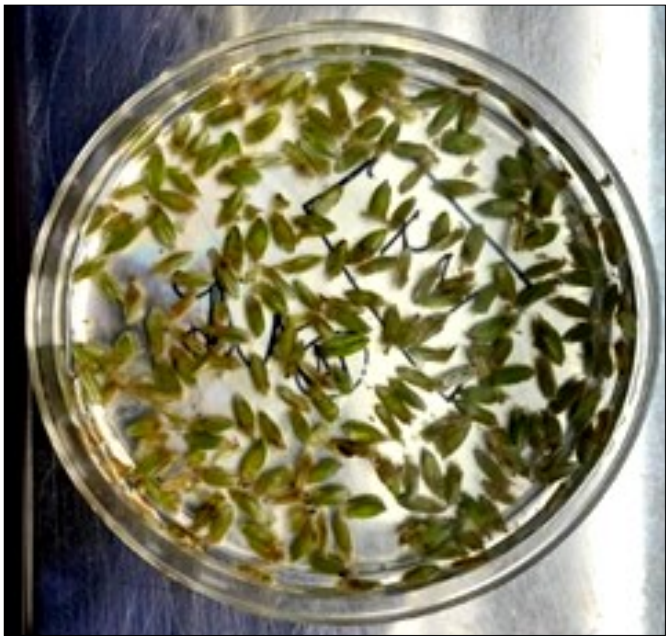

Fig. 2. Pseudoseed obtained from pollinated spikes.

because there was no morphological difference between seeds carrying embryos and those without embryos. The embryos were taken out from the pseudo seeds (Fig. 3). Embryos were visible when seeds were seen from below with a 60 W lamp overhead. This technique solved the problem of identifying embryos, which were not seen from above due to their settlement at the bottom of watery endosperm.

Dissected embryos were placed on a hormone-free MS (Murashige and Skoog) medium in 100 Petri dishes (Fig. 4). The haploid embryos were cultured and incubated in the dark for 4-6 weeks at 25°C. Subculturing of embryos was done every 3-4 weeks. When small roots and shoots were visible (Fig. 5), the embryos were shifted to the light incubator for 1-4 weeks at 25°C, 16 h light and 8 h dark conditions until they developed into green, healthy plantlets (14).

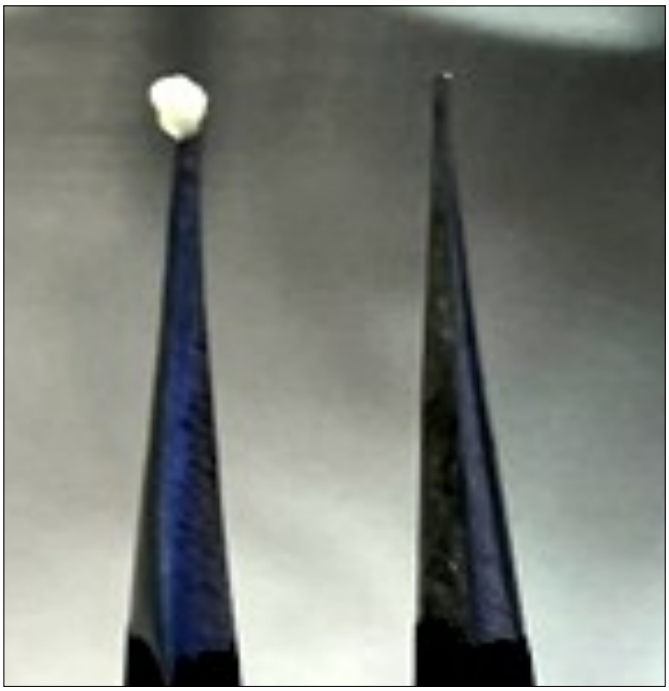

Fig. 3. Embryos obtained from the pseudoseed.

Fig. 4. Embryos are kept in media for culturing.

The following observations regarding haploid induction parameters were expressed as percentages:

$$\text{Pseudo seed formation frequency (\%)} = \frac{\text{Number of pseudo seeds formed}}{\text{Total number of florets pollinated}} \times 100 \quad \text{...(Eqn. 1.)}$$

$$\text{Embryo formation frequency (\%)} = \frac{\text{Number of pseudo seeds carrying embryos}}{\text{Total number of pseudo seeds formed}} \times 100 \quad \text{...(Eqn. 2.)}$$

Statistical analysis

Based on their individual data of embryo formation frequency, the means of the two groups-T_1 and T_2, T_1 and T_3, T_1 and T_4, T_2 and T_3, T_2 and T_4, T_3 and T_4-were compared using t-test. The means of different group pairs (T_1, T_2, T_3 and T_4) showed statistically significant differences overall, according to the t-tests. A thorough evaluation of the comparisons undertaken is made possible by the precise values given for the mean, standard deviation, t-test statistic, p-value and significant test.

Results

In the current research, two auxins, 2, 4-D and dicamba, were found to positively influence the delay in seed abortion. The compounds studied include synthetic auxin 2,4 -D, $AgNO_3$ and dicamba, applied in four different treatments (T_1, T_2, T_3 and T_4) to assess their impact on haploid embryo production and plant regeneration rates. Importantly, treatments T_3 and T_4 were found to be superior in encouraging haploid embryo production as well as the formation frequency of plants, considering that

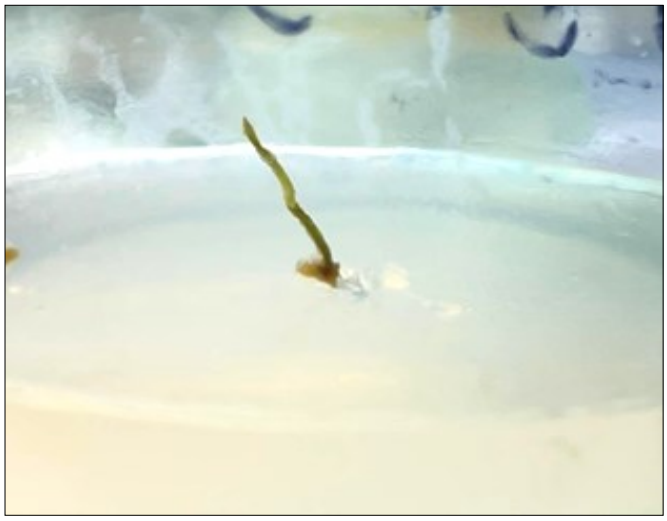

Fig. 5. Regenerated plants from embryo.

they involved 100 mg/L + 85 mg/L of 2,4-D and dicamba as well as 100 mg/L + 100 mg/L concentrations, respectively.

Effects of 2, 4-D, $AgNO_3$ and dicamba combinations on pistils pollinated with maize pollen

The phenomenon of auxin-induced parthenocarpy is widely acknowledged and demonstrated in unfertilized maize and bread wheat ovaries (15-17).

Pollination with radiation-exposed pollen can result in the development of parthenocarpic (seedless) fruits in triticale plants. The pollen does not fertilize the ovule in these situations, but it can still develop and reach it.

A combination of 2, 4-D and dicamba showed more embryo formation and plant regeneration efficiency than when 2, 4-D and $AgNO_3$ were used together. The correlation between the concentration of 2, 4-D and the enlargement of the ovary is direct (17). Principally, T_3 (2, 4-D + dicamba; 100 mg/L + 85 mg/L) showed a significant fraction (31.46%) of embryos in haploids, while the treatment exhibited 11.11% of plant formation. On the other hand, T_1 (2,4-D + $AgNO_3$ at 100 mg/L + 60 mg/L) showed inefficiency, as shown by its low frequency and efficiency.

Using silver ions in the form of $AgNO_3$ and other solutions consisting of salt of silver can prevent ethylene that is externally applied from acting on whole plants or parts of plants (18). In plants, auxins may promote ethylene and the level of internal auxins could be responsible for the rate at which ethylene is produced (19, 20). Maybe $AgNO_3$ restrains the effect of ethylene production when 2, 4-D is given externally.

Effect of auxin treatment on embryo formation frequency

Out of all four treatments, T_3 and T_4 (2,4-D + dicamba at 100 mg/L + 85 mg/L and 2,4-D + dicamba at 100 mg/L + 100 mg/ L) were reported to have more haploid embryo production, 31.46% and 30.61%, respectively (Table 1).

The t-test was conducted to compare the means of the two groups, T_1 and T_2, T_1 and T_3, T_1 and T_4, T_2 and T_3, T_2 and T_4, T_3 and T4 based on their respective data of embryo formation frequency. Overall, the t-tests reveal statistically significant differences between the means of various pairs of groups (T_1, T_2, T_3 and T_4). The specific values provided for

Table 1. Mean values of embryo formation frequency.

	T_1	T_2	T_3	T_4
IC642661	11.57	15.95	29.80	29.03
IC642662	19.75	18.51	30.85	28.37
IC642723	14.28	21.42	29.88	30.61
EC490146	17.20	22.33	30.08	28.57
EC490149	20.68	22.85	30.95	27.67
EC490148	19.31	21.83	31.46	29.89

the mean, standard deviation, t-test statistic, p-value and significant test allow for a detailed interpretation of the comparisons made (Table 2 and Fig. 6).

Based on the results presented in Table 3, the analysis indicates a significant variation in data regarding the effects of different treatments used in the research for haploid embryo formation frequency. This depicts that the treatments are equally good and affect the haploid embryo formation frequency differently concerning each other.

Effect of auxin treatment on plant regeneration frequency

The findings demonstrated in Table 4 that T_3 (2,4-D + dicamba; 100 mg/L + 85 mg/L) and T_4 (2,4-D + dicamba; 100 mg/L + 100 mg/L) showed a plant regeneration frequency of 11.53% and 11.11%, respectively. This was significant in causing the formation of haploids in distant crosses between triticale and the plants from the *Zea* genera.

The t-test was conducted to compare the means of the two groups, T_1 and T_2, T_1 and T_3, T_1 and T_4, T_2 and T_3, T_2 and T_4, T_3 and T_4 based on their respective data on plant regeneration frequency. Overall, the t-tests reveal significant differences between the means of specific pairs of groups T_1, T_2, T_3 and T_4 (2,4-D + $AgNO_3$ at 100 mg/L + 60 mg/L, 2,4-D + $AgNO_3$ at 100 mg/L + 80 mg/L, 2,4-D + dicamba at 100 mg/L + 85 mg/L, 2,4-D + dicamba at 100 mg/L + 100 mg/L) but in the group T_1 and T_2, T_3 and T_4 (2,4-D + dicamba at 100 mg/L + 85 mg/L and 2,4-D + dicamba at 100 mg/L + 100 mg/L) show no significant differences (Table 5 and Fig. 7).

Based on the results in Table 6, the analysis indicates a highly significant variation in plant regeneration frequency among the effects of different treatments used in the research for plant regeneration frequency. This depicts that the treatments are equally good and affect the plant regeneration frequency differently concerning each other.

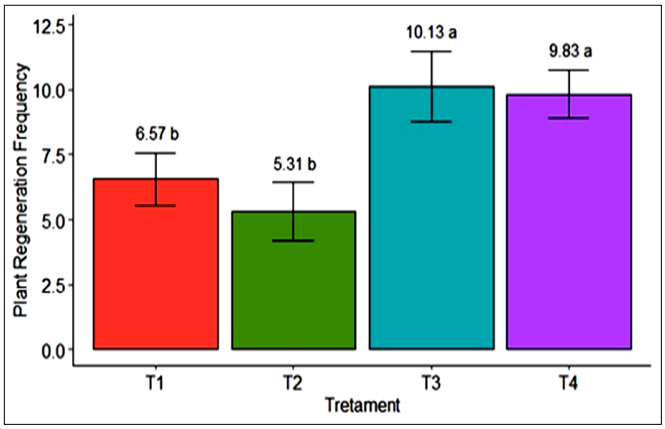

Fig. 6. Average number of haploids formed by the effect of different treatments (lower case letters show the significant difference among the treatments).

Table 4. Mean values of plant regeneration frequency.

	T_1	T_2	T_3	T_4
IC642661	7.14	6.66	9.67	11.11
IC642662	6.25	6.66	10.34	9.52
IC642723	8.33	4.76	7.69	10
EC490146	6.25	4.34	10.81	8.33
EC490149	5.55	4.16	11.53	9.67
EC490148	5.88	5.26	10.71	10.34

Discussions

The hybridization of wheat with maize results in the production of wheat haploids. This is attributed to the complete elimination of maize chromosomes from the hybrid embryos of wheat × maize (21). Likewise, the crosses between triticale and maize give rise to triticale haploids (6, 10, 22). The triticale × maize crosses exhibit lower dependency on genotype compared to androgenic methods. As a result, these crosses could be suggested in cases where the genotype displays resistance to anther and microspore

Table 2. Treatments compared for embryo formation frequency.

	Mean	Standard deviation	Correlation coefficient (r)	t-Statistic	Probability value (p-value)	Significant test
T_1	17.13	3.55	0.603633	-2.84216	0.036155	Significant
T_2	20.48	2.68				
T_1	17.13	3.55	0.848127	-10.8883	0.000114	Significant
T_3	30.50	0.67				
T_1	17.13	3.55	-0.48189	-6.96675	0.000937	Significant
T_4	29.02	1.06				
T_2	20.48	2.68	0.381554	-9.78043	0.00019	Significant
T_3	30.50	0.67				
T_2	20.48	2.68	-0.00794	-7.21183	0.000799	Significant
T_4	29.02	1.06				
T_3	30.50	0.67	-0.21862	2.615697	0.047343	Significant
T_4	29.02	1.06				

Table 3. Analysis of variance for embryo formation frequency.

Source	Degree of freedom	Sum of square	Mean sum of square	F Statistic (F-value)	Probability value (Pr < F)
Treatment	3	760.28	253.42	59.05	1.543e-08***
Genotype	5	43.1	8.62	2.00	0.1356
Error	15	64.37	4.29	-	

Table 5. Treatments compared for plant regeneration frequency.

	Mean	Standard deviation	Correlation coefficient (r)	t-Statistic	Probability value (p-value)	Significant test
T_1	6.56	1.01	0.154999	2.220943	0.077037	NS
T_2	5.31	1.11				
T_1	6.56	1.01	-0.98197	-3.72062	0.013703	Significant
T_3	10.12	1.34				
T_1	6.56	1.01	0.300353	-6.94892	0.000948	Significant
T_4	9.83	0.92				
T_2	5.31	1.11	-0.17119	-6.26181	0.001524	Significant
T_3	10.12	1.34				
T_2	5.31	1.11	0.545889	-11.2184	9.83E-05	Significant
T_4	9.83	0.92				
T_3	10.12	1.34	-0.32099	0.392789	0.710663	NS
T_4	9.83	0.92				

Table 6. Analysis of variance for plant regeneration frequency.

Source	Degree of freedom	Sum of square	Mean sum of square	F Statistic (F value)	Probability value Pr (<F)
Treatments	3	102.942	34.314	24.6318	4.785e-06 ***
Genotype	5	3.739	0.748	0.5368	0.7455
Error	15	20.896	1.393	-	

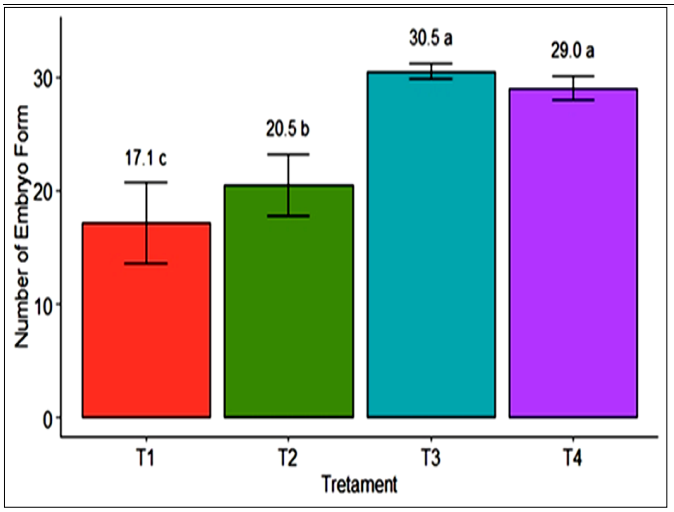

Fig. 7. Average number of plants regenerated by the effect of different treatments (lower case letters show the significant difference among the treatments).

culture (6, 23). In triticale, the occurrence rate of spontaneously generated DHs through androgenesis-inducing techniques is comparatively modest, ranging between 5% and 58% (24-30).

The above research study suggests that 2,4-D and dicamba (2,4-D + dicamba at 100 mg/L + 85 mg/L and 2,4-D + dicamba at 100 mg/L + 100 mg/L) combinations were acting significantly for embryo formation frequency and plant regeneration. Both auxins were found to be equally effective in a study assessing the effects of dicamba and 2,4-D on wheat-maize crosses at dosages of 20 mg/L and 80 mg/L. To achieve the intended results, these auxin analogues effectively took the place of the previously employed IAA or 2,4-D (10, 31-35). It also aligns more closely with a frequency of 29.8 embryos per 100 ovaries documented through anatomical examinations of pistils fixed shortly after pollination (32).

The 2,4-D and $AgNO_3$ (2,4-D + $AgNO_3$ at 100 mg/L + 60 mg/L, 2,4-D + $AgNO_3$ at 100 mg/L + 80 mg/L) combinations, did not exhibit any significant effects in the present study. However, earlier research involving post-pollination treatment using 2,4-D alone or combined with $AgNO_3$ has shown that haploid seedlings were generated from 1.7% and 3.3% of pollinated florets in two durum cultivars (33). In the case of wheat × maize, 2,4-D at 100 ppm concentration injected in plants 24, 48 and 72 h after pollination shows 47.27% embryo formation frequency in genotype WH 1105 (34).

Plants derived from interspecific hybridization between triticale and maize exhibited vigorous, green phenotypes and haploidy. This parallels the outcome observed in crosses involving wheat and maize, wherein only a singular occurrence of spontaneous chromosome duplication has been documented (35). This deviates from typical androgenesis outcomes, where occurrences of albino plants, aneuploids and spontaneously DHs are commonly reported (36, 37).

Conclusion

In conclusion, the study demonstrated that the combination of auxins, 2,4-D and dicamba, specifically at concentrations of 100 mg/L + 85 mg/L and 100 mg/L + 100 mg/L, significantly influenced embryo formation and plant regeneration frequencies in triticale × maize crosses. These concentrations proved superior to other treatments, emphasizing their potential for improving haploid embryo production and subsequent plant regeneration rates. The research findings also highlight the importance of carefully selecting auxin combinations for efficient and successful hybridization techniques in crop breeding. The study contributes valuable insights into optimizing protocols for haploid embryo production in triticale × maize crosses. These findings also lay the groundwork for improving procedures and customizing auxin treatments for particular genotypes and cross combinations. Based on this understanding, future research can investigate new auxin combinations, evaluate their suitability for a variety of crop species and improve haploid embryo development and plant regeneration. Researchers can help create more solid and dependable methods for promoting plant breeding and crop enhancement projects by expanding this methodology.

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Authors' contributions

S, PKS, SSS, PW and SS were involved in the conceptualization of the study. The study methodology was devised by S, SSS and PW. SSS and PW were involved in software validation and formal analysis. Investigation for the study was carried out by S, PKS, SSS and PW. Original draft preparation of the manuscript was done by S, SSS, PW and SS. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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