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ADVANCING *IN VITRO* PROPAGATION OF TRUE-TO-TYPE LINES OF AMARANTHUS SPP. EMPLOYING SSR-BASED GENETIC EVALUATION

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ABSTRACT

Amaranthaceae includes the genus Amaranthus, characterized by the enormous morphological and biological diversity that has allowed it to thrive in many diverse environments. Therefore, the development of transgenics relies largely on the ability to produce true-to-type plants using an efficient in vitro regeneration protocol. This study was conducted partly to develop a robust in vitro regeneration protocol for Amaranthus lines and partly to determine the reported protocol's usefulness by analyzing the regenerated plantlets' genetic diversity. Growth of the plants was most favorable with a sucrose concentration of 30g/L. A-20 and A-21 lines produced the highest callus induction response when 2,4-D and kinetin were used as plant growth regulators at 2.3µM concentration. Callus was initiated from epicotyl explants on MS medium with 2,4 D and kinetin. Callus induction rates from different lines varied, where in A-21 and A-20 100%, in A-42 82% and A-96 58%. Callus responded well to two media combinations: one being NAA (0.5µM) and Kinetin (2.3µM), and the second was Zeatin (4mg/L) and IAA (0.01mg/L). Simple sequence repeats (SSR) markers were then employed to determine whether genetic diversity exists among regenerated plants of the corresponding Amaranthus line A-20, A-21, A-42, and A-96. Genetic similarity between all lines and lack of detectable variation was evident by SSR-PCR amplification of genomic DNAs with three primer sets, AHF1+AHR1, AHF2+AHR2, and AHF3+AHR3. This study describes a reliable in vitro regeneration system for true-to-type plant development. Thus, the reported methodology provides the tools for future Amaranthus genetic studies and breeding programs.

***Keywords*:** *In vitro*, Callus, Regeneration, SSR marker, Phytohormones.

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1. INTRODUCTION

Amaranth (Amaranthus spp.) is a crop with a wide range of potential uses and high tolerance to adverse conditions of the growing environment (Venskutonis and Kraujalis 2013). Amaranth has been traditionally considered a pseudocereal and has attracted the interest of researchers worldwide due to its ability to grow in various conditions, including drought, less fertile soil, and high temperatures (Capriles et al. 2008; Shukla et al. 2010). This resilience and its ideal nutritional value have made it an attractive crop for cultivation in areas affected by climate change, and on suboptimal soils. Of the Amaranthus species, *A. caudatus*, *A. hypochondriacs*, *and A. cruentus are considered purpose crops that provide both seeds and leaves, which are good sources of protein, essential amino acids*, and micronutrients (Joshi and Rana 1991; Mlakar et al. 2010; Osuna-Ruíz et al. 2023). These characteristics improve the nutritional quality of amaranth, thus recommending it for human consumption and animal feed. Squalene, tocopherols, and polyphenols in amaranth also create the possibility of the grain being used in the nutraceutical and pharmaceutical sectors (Becker et al. 1981). Its adaptability to biotic and abiotic stress and

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short growing period make amaranth a potential "golden" vegetable crop of high economic value (Coimbra and Salema 1994; Mustafa et al. 2011; Hoidal et al. 2020). From a biologist's view, the betalains and pigments that are found in amaranth have antioxidant and anti-inflammatory properties which are useful in foods, cosmetics and medicines (Stintzing and Carle, 2004; Trejo-Tapia et al. 2008). Therefore, more research still has to be conducted to find improved genotypes with high yield potential and better resistance to both biotic and abiotic stress. Advances in tissue culture and micropropagation have shown the potential for rapid multiplication, particularly through somatic embryogenesis and organogenesis, aiding in the development of improved cultivars and the effects of in vitro digestion on the digestibility and antioxidant activity of amaranth proteins, emphasizing the potential to enhance nutritional profiles through advanced biotechnological techniques (Bennici and Schiff 1997; Serena-Romero et al. 2023).

The primary objective of this study was to develop a successful and efficient *in vitro* regeneration method for *Amaranthus* species. *Amaranthus* species are highly valued for their high protein content and balanced amino acid profile, with approximately 70 species, including grain, vegetable, and weedy types. By utilizing different types of explants, such as calluses, leaves, epicotyl, and stems, the ultimate research aim was to identify the most suitable methods for enhancing the regeneration potential of *Amaranthus*, directly contributing to the improved cultivation and utilization of this important crop. A method involving a hypocotyl-based regeneration, using a medium containing growth regulators (e.g. kinetin and 2,4-dinex, to form callus and promote plant regeneration, was previously reported (Kumam et al. 2024). A combination of plant growth regulators, including benzylaminopurine (BAP) and naphthaleneacetic acid (NAA), has been found to optimize organogenesis in several crops. These findings are vital for enhancing the regeneration systems of Amaranthus species, developing more effective micropropagation methods, and making better use of the industrial value of (Locy and Fisher 1985; Xuan et al. 2023).

Moreover, the genetic diversity in plant species has to be determined to avoid unwanted mutations in plants regenerated in vitro, particularly for plant regeneration through callus induction without the final aim of developing transgenics (Ray and Roy 2007). Analysis of genetic diversity and the elucidation of evolutionary relationships of cultivated and wild Amaranthus species have become dependent on molecular markers such as simple sequence repeats (SSRs), random amplified polymorphic DNA (RAPDs), and amplified fragment length polymorphism (AFLPs) (Trucco and Tranel 2011). These markers are highly reliable, unaffected by environmental conditions, and therefore ideally suited for genetic selection and breeding programs (Kalendar and Glazko 2002; Zhao et al. 2011). PCR based DNA markers are extremely powerful in detecting inter and intra-species variations and also in studying population structure, gene flow and genetic mapping (Li and Park 2012; Slotta 2008; Tam et al. 2005).

Being the first work along this line, this current study is therefore directed towards the establishment of efficient in vitro regeneration methods and the use of molecular markers such as SSRs to assess genetic diversity. In this study, different explant types and different growth regulator combinations for new shoot regeneration were explored and optimized to develop an efficient in vitro regeneration system; the genetic diversity of Amaranthus species was assessed through the utilization of molecular markers to identify proper candidates for selection and to reduce undesirable mutations in regenerated plants. These advancements are pivotal for improving amaranth's adaptability, enhancing its commercial value and supporting breeding programs to cultivate superior varieties with higher yields and stress tolerance in general for the plant scientific community.

2. MATERIALS AND METHODS

2.1. Plant Material

Seeds of the Amaranthus species A-20, A-21, A-42 and A-96 lines were collected from the Center of Agricultural Biochemistry and Biotechnology, University of Agriculture Faisalabad. Four lines of *Amaranthus* spp. were used in this study. These are A-20, A-21, A-42 and A-96, respectively.

2.2. Sterilization of Seeds and Culturing on Media Plates

To prepare the culture medium, 4.33g of MS salts and 30g) were dissolved in 900mL distilled water, adjusted to 1 liter and the pH was set to 5.8. Gellan gum (2.6g) was added separately and the medium was autoclaved at 121°C and 15psi. The seeds were sterilized with 70% ethanol and mercuric chloride, rinsed thoroughly, and cultured on sterile MS0 plates. Approximately four to five seeds from four Amaranthus varieties were cultured on plates under sterile conditions in UV laminar flow. Ethanol and a Bunsen burner were used to maintain sterility. Plates were incubated in a growth room at 25°C with a 16-hour light/8-hour dark cycle, ensuring optimal germination conditions. Thoroughly washing seeds with distilled water after sterilization is a common practice to mitigate these adverse effects, ensuring a higher germination rate and healthier seedlings (Mohtasim and Islam 2023). In the other experiments, no protocol was followed. Distilled water sterilized the surface of the Amaranth seeds.

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2.3. DNA Isolation of Amaranthus

The cetyltrimethylammonium bromide (CTAB) – isopropanol precipitation method (Paterson et al. 1993) was used to isolate genomic DNA.

2.4. Seed Germination and Callus Induction

Murashige and Skoog medium supplemented with 3, 4.5, 6.0, 7.5 and 9.0% sucrose was prepared for seed germination and analysis of the effect of sucrose on plant growth. The media were prepared following Bennici and Schiff (1997). In a rinsed 1-liter beaker, 900mL of distilled water was mixed with 4.43g MS salts, 30g sucrose, 1.5mL of 1M 2,4-D, and 1.5mL of 1M kinetin on a magnetic stirrer. The pH was adjusted to 5.8, and the volume was 1 liter. Gellan gum (2.8 g) was added to the glass bottle before the mixture was autoclaved. After sterilization, the medium was poured into 35–40 petri plates under sterile conditions.

2.5. Indirect *in vitro* Regeneration of *Amaranthus* using Combinations of Plant Growth Regulators

Distilled water (900mL distilled placed in a glass beaker and rinsed with distilled water for cleaning, and approximately 500mL distilled water was added. Then added 2.658g/600mL MS salts and 18g/600mL sucrose were added, and the mixture was placed on a magnetic stirrer for mixing. We took 1.44mL/600mL of NAA from the prepared stock solution and 2.64mL/600mL of BAP from its stock solution, which were added to the medium. After mixing the ingredients in water, the pH was set to 5.8, making up a volume of 1 liter. Gellan gum powder was not added to the beaker, although it was added to a 1L distilled glass bottle with a quantity of 1.68g. At the end, pour all media in a glass bottle and shake for 1 to 2min. The final solution was one liter. Subsequently, the whole medium was autoclaved before pouring into Petri plates. When the medium was autoclaved, it was poured into the plates.

2.6. Regeneration Media have a Combination of IAA and BAP

The recipe for the regeneration medium, designed for a 1000mL solution, includes the following components: 30g/L of sucrose as the primary carbohydrate source, 4.43g/L of Murashige and Skoog (MS) salts to provide essential nutrients, and 2.8g/L of gellan gum powder for gelling the medium. In addition, 500mg/L casein hydrolysate was added to promote tissue growth. To further enhance the medium, 1mg/L of pyridoxine and nicotinic acid were included as vitamins, while 0.1mg/L of ascorbic acid serves as an antioxidant. For plant growth regulators, 3mg/L of BAP (6-benzylaminopurine) was used for its cytokinin properties, and 1mg/L IAA (Indole-3-acetic acid), an auxin. To optimize the environment for plant tissue regeneration, pH of the medium was adjusted to 5.8 to optimize the environment for plant tissue regeneration.

2.7. Regeneration Media having Combination of NAA and Kinetin

The regeneration medium protocol for 1000mL contained 30g/L of sucrose, a carbon source to support plant growth. 4.43g/L of Murashige and Skoog (MS) salt solution was also added for provision of essential nutrients, as well as 2.8g/L of gellan gum powder to solidify the medium. With care, the pH was adjusted to 5.8 as an environment in which to grow plant tissue culture. It contains 0.5µM NAA naphthaleneacetic acid (NAA; an auxin that promotes root formation) and 2.3µM kinetin (a cytokinin promoting shoot and cell division).

2.8. Combination of BAP and Kinetin

The protocol for the regeneration medium, intended for a 1000mL solution, consists of several key components. It includes 30g/L of sucrose to supply the necessary energy for plant tissues and 4.43g/L of Murashige and Skoog (MS) salts to provide essential macro and micronutrients. 2.8g/L gellan gum powder was added to solidify the medium, ensuring a suitable texture for plant tissue growth. The pH was adjusted to 5.8, creating an optimal environment for plant development. Additionally, the medium contains 0.5mg/L of BAP (from a 1 Molar stock solution) and 0.5mg/L of kinetin (also from a 1 Molar stock solution), both of which are plant growth regulators—BAP being a cytokinin that promotes shoot formation, while kinetin aids in cell division and overall tissue regeneration.

2.9. Combination of Zeatin and IAA

The regeneration medium for a 1000mL solution includes several vital components for supporting plant growth and tissue development. The medium contained 30g/L sucrose, the primary energy source, and 4.43g/L Murashige and Skoog (MS) salts to provide essential nutrients. To solidify the medium, 2.8g/L of gellan gum powder is added. The pH was carefully adjusted to 5.8, to ensure an optimal environment for plant tissue regeneration. Additionally, 4mg/L of zeatin, a cytokinin, was added to stimulate shoot formation and cell division. The medium also contains 0.01mg/L IAA (Indole-3-acetic acid), an auxin that promotes root growth, and 0.2mg/L of GA3 (Gibberellic acid) to enhance cell elongation and overall plant growth.

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2.10 Combination of Zeatin and IAA

The recipe for the regeneration medium, formulated for a 1000mL solution, includes 30g/L of sucrose as the primary carbohydrate source, and 4.43g/L of Murashige and Skoog (MS) salts to provide the essential nutrients for plant growth. To solidify the medium, 2.8g/L of gellan gum powder is added. The pH was adjusted to 5.8 to create a conducive environment for plant tissue culture. Additionally, 5mg/L of zeatin, a cytokinin, was added to promote shoot formation and cell division. The medium also contains 0.01mg/L IAA (Indole-3-acetic acid) to stimulate root growth, and 0.2mg/L of GA3 (gibberellic acid) to promote cell elongation and enhance overall plant development.

2.11. Combination of Media with Zeatin

The regeneration medium recipe, prepared for a 1000mL solution, includes 30g/L of sucrose as the primary energy source and 4.43g/L of Murashige and Skoog (MS) salts to supply essential nutrients. To solidify the medium, 2.8g/L of gellan gum powder was used. The pH was adjusted to 5.8, ensuring an optimal plant tissue growth environment. Additionally, 2mg/L zeatin, a cytokinin, was incorporated to promote cell division and shoot formation during regeneration.

2.12. Acclimatization

A healthy, developed, and fixed bud was taken aseptically, and the roots were rinsed using running tap water so as to separate it from the MS basal media bound to it. The seedlings were then transferred into the soil, which consists of topsoil and cow dung in a ratio of 2:1, in polythene bags. They were then transferred into a larger transparent polythene bag and water was tipped on the bag before air was blown into the bag. The mouth of the bag was tied and hung on the rafter of the outside room (Tahir and Mathew 2021).

2.13. SSR Primers used in this Study (Table 1)

Table 1: SSR primers for PCR analysis (Lee et al. 2008)

Sr. #	Forward primer	Reverse primer
1	TGAATCCTTATGCGCCAC	GGAGCCCTGTCCTCATGT
2	CTCCTCGGGAGAAGGTTG	TGTGTCCCAATCCATCGT
3	GAGGAGACTTGGTGGCCT	TCGGGAGCAATGTAGCAC

3. RESULTS

3.1. Effect of Surface Sterilization Protocol on Germination of Amaranthus

3.1.1. Effect of Mercuric Chloride and 70% Ethanol on Germination of Seeds: This combination of seed sterilization causes severe damage to seeds. This suggests that the seeds were highly sensitive to the sterilization process. Mercuric chloride, a potent and disruptive chemical, is known to cause seed germination when used for sterilization.

3.1.2. Effect of Double Distilled Water on Germination of Seeds: The results showed the germination percentage of seeds from four different genotypes, labeled A-20, A-21, A-42, and A-96, based on the number of cultured seeds. For genotype A-20, 13 out of 20 seeds germinated, resulting in a germination rate of 46%, which was notably lower than that of the other genotypes. In contrast, genotypes A-21 and A-42 had seven seeds cultured, with five of them successfully germinating in each case, leading to a higher germination rate of 71%. The highest germination rate was observed in genotype A-96, where five out of six seeds germinated, giving an impressive germination rate of 83% (Fig. 1E). These results suggest that genotype A-96 has the best ability to germinate under the conditions provided, whereas genotype A-20 shows comparatively lower germination success, indicating that either genetic factors or the specific environmental conditions used may have been less favorable for this genotype. The varying germination percentages among the genotypes highlighted the influence of genetic variability on the germination potential of the seeds (Fig. 1A-D).

3.2. Callus Induction Difference Among Various Varieties

The callus induction response by the A-20 and A-21 lines was approximately 100% (Fig. 1F, I). After that, at the 3rd number, callus induction was observed in line A-42 (Fig. 1G) at about 82%. The last, A-96, showed 58% callus induction (Fig. 1H). The results presented provide information on the characteristics of callus formation for four genotypes (A-96, A-21, A-42, and A-20), focusing on callus color, type, and shape. Across all genotypes (A-96, A-21, A-42, and A-20) (Table 2), callus color was consistently described as yellowish, indicating no color variation between these genotypes under the conditions used in the experiment. Yellowish calli are common and often associated with healthy, viable calli in plant tissue culture (Table 2). In terms of callus type, all four genotypes had compact calluses. A compact callus is typically dense, firm and tightly packed, which often indicates a more

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organized growth pattern. Compact callus formation is an important trait in tissue culture, as it is associated with a higher potential for regeneration and development of shoots or roots from the callus.

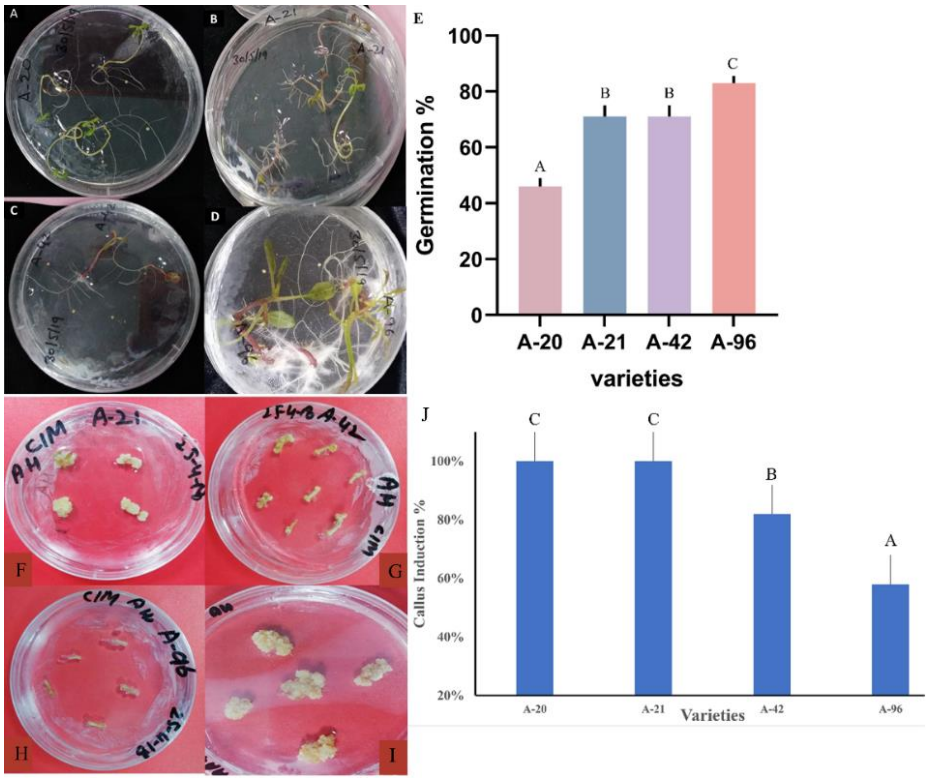

Fig. 1: Germination of Seeds: A (A-20), B (A-21), C (A-42) and D (A-96), and E graphical analysis were obtained without any sterilization protocol. Illustrates callus induction in various *Amaranthus* species under *in vitro* conditions Variety A-21 (F) and A-21(I) exhibits the highest level of callus formation, with varieties A-42 (G) and A-96 (H) showing relatively lower callus development. The bar graph (J) further quantifies the callus induction percentage, demonstrating that A-20, A-21 achieved the highest induction rate, nearing 100%, while A-42 and A-96 showed significantly lower percentages. Error bars represent the standard deviation across experiments, highlighting the variability in response between the different varieties.

Table 2: Morphology of Callus

Genotype	Callus Color	Callus type	Callus Shape
A-96	Yellowish	Compact	Granular
A-21	Yellowish	Compact	Granular
A-42	Yellowish	Compact	Granular
A-20	Yellowish	Compact	Granular

The callus shape for all four genotypes was described as granular, indicating that the callus surface had a grainy or uneven appearance. Granular calli also indicate good cell division and growth potential. Granular-shaped calli often reflect a favorable environment for cellular differentiation, which is crucial for successful tissue regeneration (Table 2). The results suggested no significant variation in callus characteristics among the four genotypes tested. All genotypes exhibited yellowish, compact, and granular calli, which are generally positive indicators in tissue culture, potentially signaling the ability of these genotypes to regenerate under the conditions provided effectively. Uniformity of the results across genotypes implies that these traits are stable and consistent in these specific plant lines.

3.3. In vitro Regeneration of Amaranthus Plant

Calli was obtained from different varieties of Amaranthus. After 20 days, the obtained calli were transferred to different regeneration media. This regeneration medium was prepared using MS0 medium and NAA and BAP were added.

3.3.1. Regeneration Response of Amaranthus Lines on Multiple Media Combinations: The media combinations contained different concentrations of plant growth regulators, such as BAP (6-Benzylaminopurine), Naphthaleneacetic Acid (NAA), Kinetin, IAA (Indole-3-Acetic Acid), and zeatin. The results from each combination varied significantly, demonstrating the differential response of callus regeneration based on growth regulator concentration and specific Amaranthus genotype.

No regeneration response was observed when calli were transferred to media containing BAP 4.4µM and NAA 2.4µM. The experiment included calli of ages 7, 14, 21, and 28 days, but none of the calli across any of the genotypes responded to this hormonal combination. This suggests that the combination of BAP and NAA at the

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tested concentrations was ineffective in promoting regeneration in Amaranthus lines (Fig. 2A-C).

In another set of experiments, calli were transferred to a media containing BAP 3mg/L and IAA 1mg/L, but again, no regeneration was observed. Calli of various ages (7, 14, 21, and 28 days) failed to show any signs of regeneration on this media combination, indicating that these concentrations of BAP and IAA were not conducive to promoting the regenerative capacity of the calli in Amaranthus lines (Fig. 2D-F).

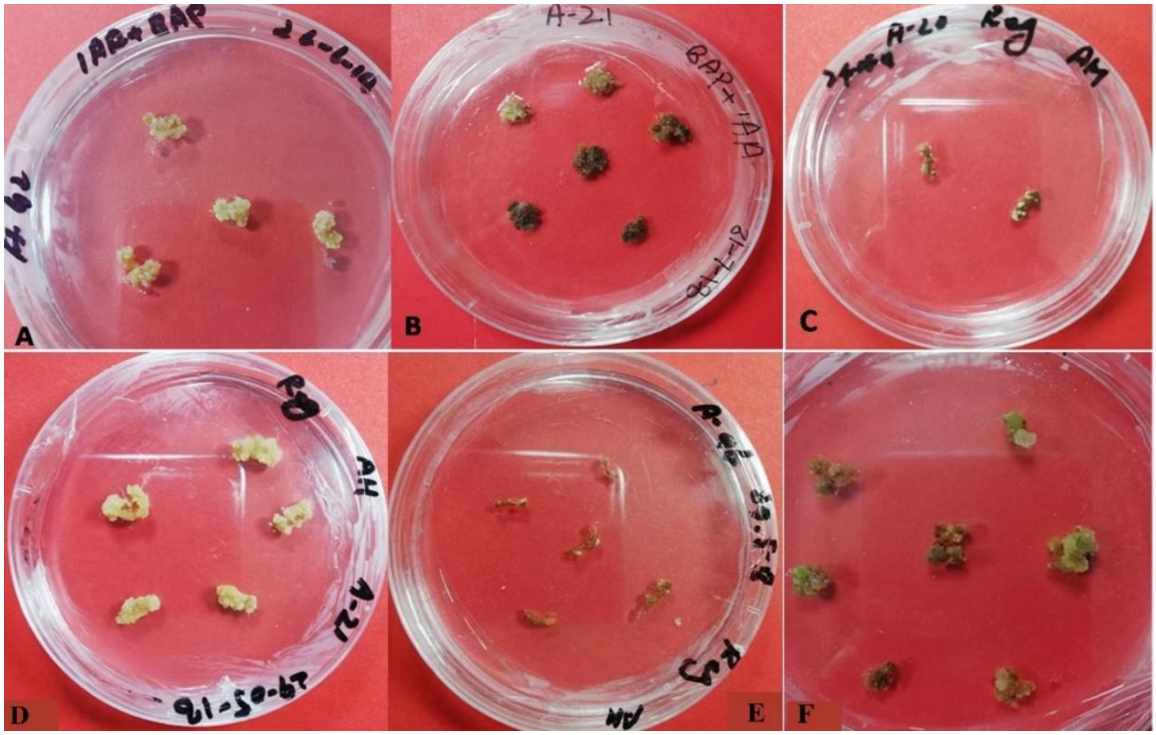

Fig. 2: Regeneration response of *Amaranthus* lines on BAP (4.4µM) and NAA (2.4µM (A-C), Regeneration response of *Amaranthus* lines on BAP (4.4µM) and NAA (2.4µM) (D-F).

Media containing NAA 0.5µM and Kinetin 2.3µM showed a very low response in terms of regeneration. Only two varieties, A-20 and A-42, exhibited signs of regeneration. Specifically, variety A-20 exhibited regeneration from the callus, whereas variety A-42 showed direct regeneration without passing through the callus phase. This suggests that this combination of NAA and Kinetin can induce regeneration in specific genotypes; however, its effectiveness is limited, and its impact varies between direct regeneration and callus-mediated regeneration (Fig. 3A-D). When calli were transferred to media containing 2mg/L zeatin, there was no regeneration response. This media combination was specifically tested for direct regeneration using hypocotyl explants, but no positive results were obtained, indicating that zeatin at this concentration was not effective in inducing regeneration in the tested Amaranthus lines (Fig. 3E-H).

Similarly, no regeneration response was recorded when calli were transferred to media containing BAP 0.5µM and Kinetin 0.5µM. Calli of different ages (7, 14, 21, and 28 days) were tested, but no positive responses were observed. This lack of response implies that this hormonal combination did not trigger regenerative processes regardless of callus age (Fig. 4A-D). However, when the concentration of zeatin was increased to 4mg/L and combined with IAA 0.01mg/L, some positive regeneration results were observed. Specifically, in variety A-20, callus regeneration was achieved, suggesting that this media combination is capable of inducing regeneration, but only in certain genotypes. This combination represents an improvement over previous trials, indicating the importance of hormone concentrations and their synergy in influencing regeneration success (Fig.4E-H).

Finally, the media containing Zeatin 5mg/L and IAA 0.01mg/L yielded the most promising results for regeneration. Nodes and internodes were used as explants in this experiment and direct regeneration was successfully achieved. This combination showed a significant improvement in regeneration efficiency, indicating that a higher concentration of zeatin combined with a low concentration of IAA is particularly effective in promoting direct regeneration in Amaranthus lines. The use of nodes and internodes as explants further facilitates the regenerative process (Fig. 5 A-D).

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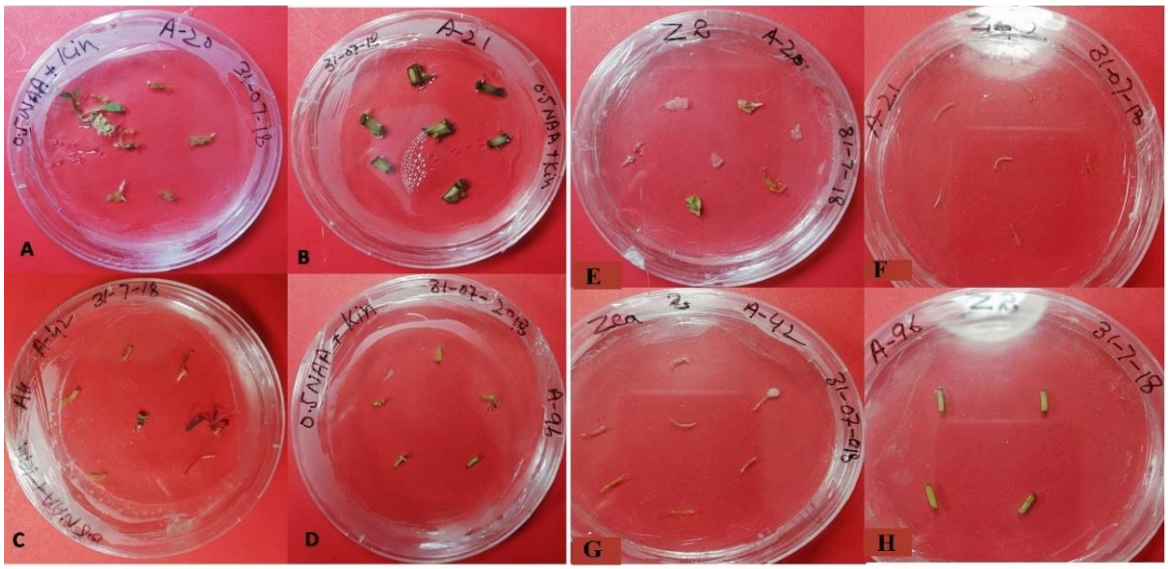

Fig. 3: Regeneration response of *Amaranthus* lines on NAA (0.5µM) and Kinetin (2.3µM) (A-D), Regeneration response of *Amaranthus* lines on Zeatin media (2mg/L) (E-H).

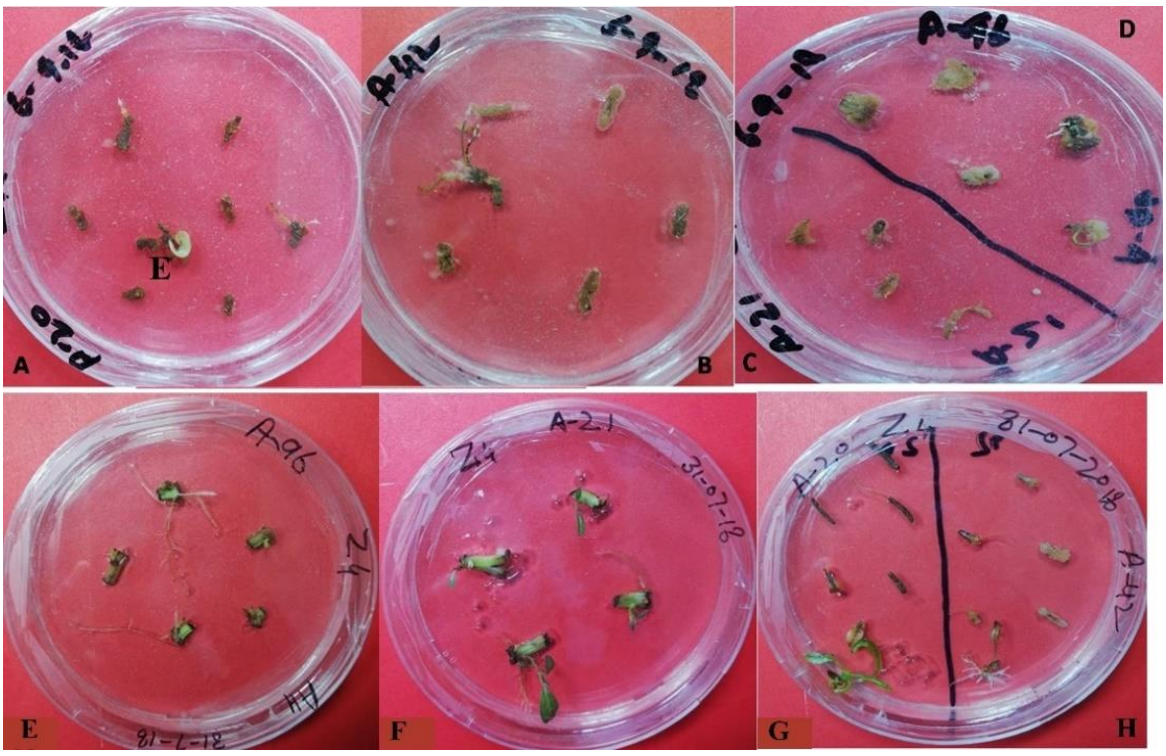

Fig. 4: The regeneration response of *Amaranthus* plant lines: Regeneration response of *Amaranthus* lines on BAP (0.5µM) and Kinetin (0.5µM) media (A-D), Regeneration response of *Amaranthus* lines on Zeatin (4mg/L) and IAA (0.01mg/L) (E-H).

In conclusion, this study demonstrates that the success of regeneration in Amaranthus lines is highly dependent on the specific combinations and concentrations of plant growth regulators. Among the tested combinations, media containing zeatin (5mg/L and IAA 0.01mg/L) proved the most effective, especially when using nodes and internodes as explants. This combination could promote direct regeneration, whereas other combinations, particularly those involving BAP and NAA, were less effective or ineffective. The differential responses observed between the genotypes further emphasize the importance of tailoring media compositions to specific plant varieties for successful regeneration.

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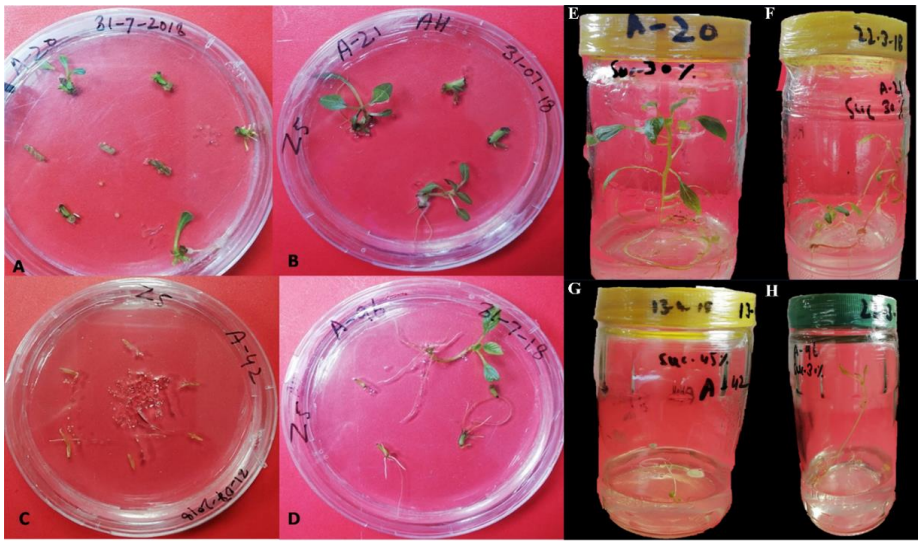

Fig. 5: Regeneration response of *Amaranthus* lines on Zeatin (5mg/L) and IAA (0.01mg/L) (A-D), Comparison of shoot length at different concentrations of sucrose (E-F).

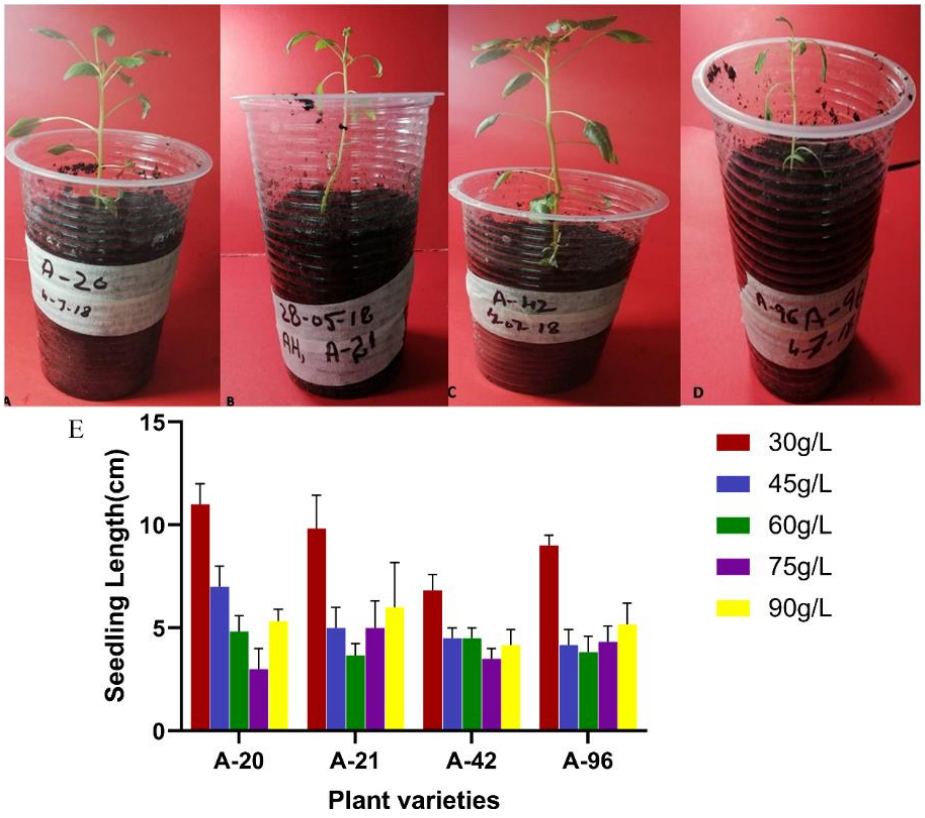

Fig. 6: Acclimatization: The plants from different genotypes (A-20, A-21, A-42, and A-96) have been shifted into small pots, as shown in the individual figures labeled A, B, C, and D, which correspond to different varieties, (E) Shoot Length of plants at different concentration of Sucrose.

3.4. Effect of Enhanced Sucrose Concentration on Plant Growth

Shoot lengths of four plant varieties (A-20, A-21, A-42, and A-96) were subjected to varying concentrations of sucrose (30, 45, 60, 75, and 90g/L). Three treatments were conducted for each variety, and shoot length was measured in centimeters to assess the influence of sucrose concentration on plant growth (Fig. 5E-H). For Variety A-20, the results indicated that the highest shoot lengths were recorded at the lowest sucrose concentration (30g/L), ranging from 10cm to 12cm across the three treatments. As the sucrose concentration increased to 45g/L and 60g/L, the shoot length progressively declined, with measurements ranging from 5cm to 8cm. At 75g/L, shoot lengths reached their lowest values between 2 and 4cm. interestingly, at 90g/L, there was a slight recovery, with shoot lengths of 5–6cm, suggesting that extremely high sucrose concentrations were less inhibitory than moderate concentrations for this variety (Fig. 6E). For variety A-21, the pattern was similar. The most extraordinary shoot lengths were observed at 30g/L, ranging from 8 to 11cm. As the sucrose concentration increased to 45g/L and 60g/L, shoot length decreased sharply, with values dropping from 3cm to 6cm. At 75g/L, moderate recovery was

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observed in some treatments, with shoot lengths reaching up to 5.5cm. At 90g/L, the shoot lengths increased even more, especially in treatment 1, where it reached 8.5cm, suggesting that this variety responded better to higher concentrations than the others (Fig. 6E).

For Variety A-42, the shoot lengths followed a similar decreasing trend as the sucrose concentration increased. At 30g/L, the shoot lengths were the highest, with values between 6 and 7.5cm. As the concentration increased to 45g/L and 60g/L, the shoot length declined to between 4 and 5cm. At 75g/L and 90g/L, growth was relatively stable but low, with shoot lengths ranging from 3.5cm to 5cm, indicating that higher sucrose concentrations had a greater inhibitory effect on this variety (Fig. 6E).

In conclusion, the best growth (in terms of shoot length) was observed across all four varieties at the lowest sucrose concentration (30g/L). As the sucrose concentration increased, shoot length generally decreased, indicating an inhibitory effect of sucrose on plant growth. However, at the highest concentration (90g/L), some varieties (A-21 and A-96) showed partial recovery, suggesting they may be more adaptable to high sucrose levels than others. These results suggest that moderate sucrose concentrations (around 45g/L to 60g/L) tend to limit growth, whereas very low or very high concentrations allow for better plant development in certain varieties.

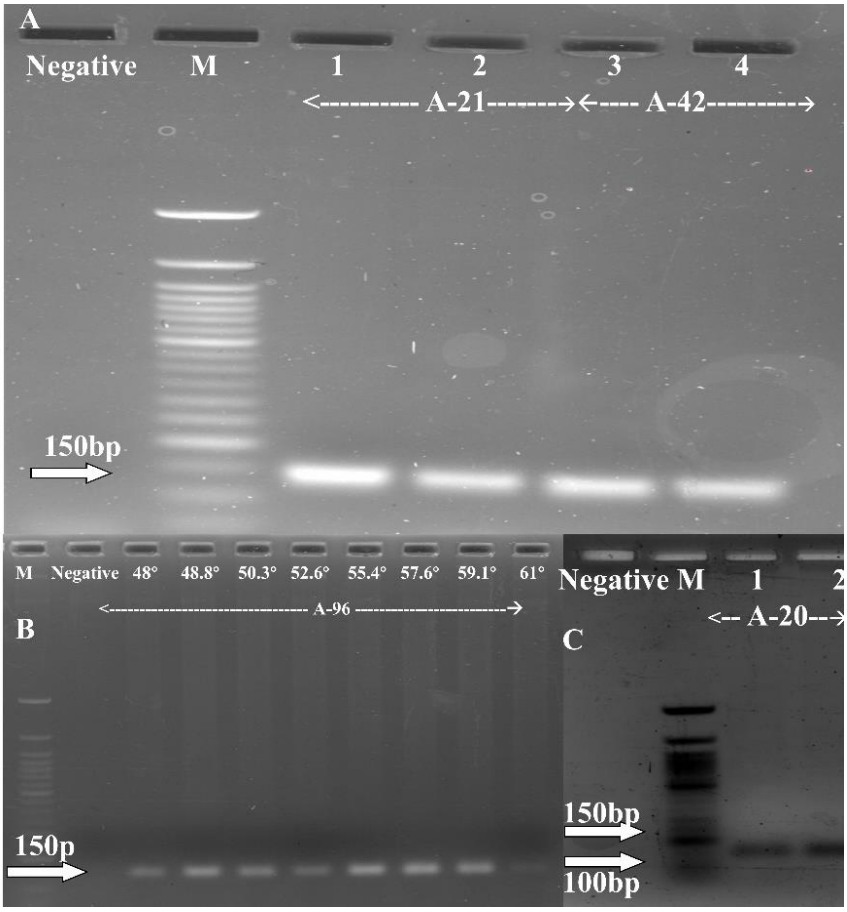

Fig. 7: SSR Marker Analysis of Genetic Diversity in Amaranth Using Agarose Gel Electrophoresis: Panel A: Agarose gel showing DNA from Amaranth samples using SSR markers. It includes a negative control, a size marker (150 bp highlighted), and lanes 1-4, A-21 to A-42, which display diverse DNA fragment patterns indicative of genetic variability. Panel B: Extended DNA analysis of Amaranth. The gel includes a negative control and a DNA ladder, with multiple bands in lanes labeled A-96 and others, showcasing the polymorphism in genetic markers. Panel C: Agarose gel electrophoresis of Amaranth DNA, with a negative control, size marker (150 bp and 100 bp), and sample lane A-20. Clear bands at 150 bp and just below 100 bp indicate specific gene amplification.

3.5. Acclimatization of Plants

3.5.1. Acclimatization: Well-grown and rooted bud was excised aseptically, and the roots were washed thoroughly with a running tap to make it free from the MS basal media attached to them. The seedlings were then transferred into topsoil and cow dung in a ratio of 2:1 in polythene bags. They were then placed in a more oversized transparent polythene bag; water was then sprinkled inside, and air was blown into the bag. The bag was tied and hanged on the rafter of the outside room (Tahir et al. 2015). At this stage, the plants from different genotypes (A-20, A-21, A-42, and A-96) were shifted into small pots, as shown in the individual Fig. 6 A, B, C, and D, corresponding to different varieties. Each plant was labeled with its genotype and the date of transfer, indicating that the plants were being closely monitored throughout the acclimatization process (Fig. 6A, B, C, D). The plants were covered with plastic (polyethylene) bags to minimize moisture loss, a critical step during acclimatization to prevent desiccation as the plants transition from a high-humidity environment (such as tissue culture) to ambient conditions. The bags help maintain a microclimate around the plant, reducing water stress and allowing the plants to gradually adapt to lower

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humidity levels. The plants were maintained in a growth room at 25±1°C, which is the optimal temperature for plant acclimatization, to promote healthy growth while minimizing stress. This controlled environment ensures that plants have consistent conditions to support their development after transfer from the in vitro phase. This image showed the successful acclimatization of regenerated Amaranthus plants from different genotypes. The uniform growth of the plants indicates that the transition to soil and environmental conditions was well managed. The use of peat moss and plastic covers, along with controlled temperature, demonstrates a careful approach to acclimatization, ensuring the plants' survival and adaptation outside of in vitro conditions.

3.6. PCR Amplification of A-21, A-96, A-20 and A-42 Lines using SSR Primers

PCR amplification of Amaranthus lines A-21, A-96, A-20 and A-42 using SSR primers AHF1+AHR1, AHF2+AHR2, and AHF3+AHR3 provided significant insights into their genetic diversity and composition. For the AHF1+AHR1 primers, amplification was conducted in a gradient temperature range of 47–57°C for lines A-21, A-96 and A-42, yielding consistent monomorphic bands of 150 bp. Line A-20 was amplified at 55°C, where a bright, distinct band of the same size was observed. A 50 bp DNA ladder was used as a size reference to ensure accurate band identification (Fig. 7A). The absence of bands in the negative control lanes confirmed the experiment's reliability by ruling out contamination or non-specific amplification. The uniformity of the 150 bp bands suggested a conserved genetic sequence across these samples. When the AHF2+AHR2 primers were used, the amplification ranged from 48 to 60°C. A-96 successfully amplified at multiple temperatures, whereas A-20 produced a bright band at 56°C. DNA fragments primarily appeared at 150 bp, demonstrating genetic uniformity among some samples, whereas others displayed polymorphic patterns, suggesting genetic variability (Fig. 7B). Including a negative control lane ensured the validity of the results, whereas the DNA marker provided precise size references. The variation in band intensities and positions reflected differences in genetic material across the analyzed lines, which is critical for assessing genetic diversity.

The AHF3+AHR3 primer set further revealed differences in the DNA sequences among the Amaranthus lines. The amplification of A-96, A-21, A-42, and A-20 was conducted at a gradient temperature range of 50–60°C. A-96, which was amplified at 58°C, showed distinct bands at 250 bp. Similarly, A-20 produces strong bands at this temperature. A 50 bp DNA ladder served as a size reference, with marker bands visible at 300 and 200 bp. Negative control lanes were used to confirm the specificity of PCR, and no contamination was detected (Fig. 7C). The samples showed bands of varying intensities and sizes, indicating the presence of multiple DNA fragments. This variability highlights genetic polymorphisms and underscores the effectiveness of the primers in analyzing genetic diversity.

Overall, PCR experiments with these three primer sets demonstrated monomorphic and polymorphic patterns among the Amaranthus lines, reflecting a balance of conserved genetic sequences and diversity (Fig. 7). The absence of amplification in the negative control lanes across all experiments validated the specificity and accuracy of the assay. These findings provide a foundation for further genetic studies, leveraging the identified markers to explore Amaranthus species' genetic relationships and diversity.

4. DISCUSSION

4.1. In Vitro Techniques for Unlocking the Genetic Promise

The aim of this study was to evaluate genetic potential of Amaranthus crop using the manipulation of MS media and the hormonal concentrations (Bewley and Black, 1994; Murashige, 1990; Uranbey et al. 2005). A successful plant tissue culture requires an established reliable and efficient plant regeneration protocol. Moreover, under in vitro conditions, differentiation and regeneration subsequently induces soma clonal variation that could otherwise be a good source for crop improvement. The seeds of the four Amaranthus lines were susceptible to the sterilization process, which employed mercuric chloride as a potent and disruptive sterilizing agent that frequently results in germination problems. Therefore, the seeds were thoroughly washed with distilled water. The A-96 germinated more than any other and had the highest germination percentage, while the A-20 had the lowest. After germination, the seedlings were transferred to jars containing different sucrose concentrations (30, 45, 60, 75, and 90g/L). Among these, 30g/L sucrose resulted in the most favorable plant growth, outperforming the other sucrose concentrations. To induce callus formation, hypocotyls of the plants were cultured on callus induction media. All lines of Amaranthus responded well to the induction process. The highest callus induction response was observed in the A-20 and A-21 lines using 2,4-D and kinetin, both at a concentration of 2.3µM. Callus morphology was consistent across all lines, with a yellow color, granular texture, and compact form. Several media combinations were tested for callus regeneration, but only two successfully induced regeneration in A-20. The first combination consisted of NAA (0.5µM) and Kinetin (2.3µM), while the second combination involved Zeatin (4mg/L) and IAA (0.01mg/L). The callus from Amaranthus can be induced from hypocotyl portions taken from aseptically germinated seeds to media containing MS salts and vitamins and supplemented with either 3 or 10mg/L 2,4-D and

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0.05mg/L kinetin. MS media supplemented with IAA or NAA and up to 10mg/L BA or kinetin did not produce a proliferating callus (Locy and Fisher 1985). Plant growth was most favorable in 30g/L sucrose, which produced greater plant growth than other sucrose concentrations. This finding is consistent with past studies, for instance, studies that proved the significance of sucrose concentration for the promotion of growth and development in different plant species (Lang et al. 2020). Carbon source sucrose and also osmotic regulation are important for seedling establishment (Avci 2019). It gives the optimal concentration to produce embryogenic calli in Porang with 2,4-D at 1mg/L and kinetin at 0mg/L which gives an intermediate callus color in yellowish–brown and wet weight at 2,43g (Agung et al. 2023). The use of Zeatin and IAA has been documented in other studies. Moreover, it is studied that the zeatin combined with other growth regulators noticeably enhanced shoot regeneration in lettuce, suggesting zeatin also works well in other plant regeneration protocols (Lim et al. 2011). This aligns with the results observed in the A-20 line of Amaranthus, where Zeatin was part of a successful media combination for regeneration. In addition of GA3, auxin and cytokinin had significantly shorten the number of days to germination, vigor and shoot length. Nardo et al. (2020) had also suggested that the hormones GA3, BAP and NAA stimulate growth in *A. annua*.

The shoot length data indicated that the highest shoot lengths were recorded at the lowest sucrose concentration (30g/L). In another study the highest shoot length was recorded in media treated with 0.15mg/L GA3 (Tahir and Mathew, 2021). It may be attributed to the effect of GA3 in stimulating early growth and development of plants by promoting cell division in epical meristem and cambium tissues (Taylor et al., 1997), similarly, shoot elongation, rate of multiplication, growth, and quality of shoots have been improved in the shoot proliferation media using GA3 (Brand and Lineberger, 1992). Across all lines, the morphology of the callus showed consistency, i.e. yellow in color, granular in texture and compact in form, which suggests healthy callus tissue (Habibah et al. 2023). Results from this study demonstrate opportunities for cultivating Amaranthus as a worthwhile crop and outline a systematic methodology for developing more efficient plant regeneration and acclimatization protocols. Many of these processes are essential for the large-scale propagation and preservation of valuable germplasms Amaranthus species have a critical role to play in meeting these agricultural food production challenges (Hobbs 2007). Amaranth is emerging as a promising food crop, with over 20 years of research and technology development driven by its valuable traits, including resistance to diseases and pests and heat and drought stress, as well as its high nutritional content in both leaves and seeds.

4.2. SSR-Based Genetic Evaluation

Because SSRs are abundant, highly polymorphic, and codominant, they are widely employed in genetic research (Gwag et al. 2010; Vignal et al. 2002). We use SSR markers to study the genetic diversity of four Amaranth lines (A-20, A-21, A-42, A-96). Genetically and morphologically, the genus Amaranthus includes approximately 70 varied species(Ray and Roy, 2007)Four lines were subjected to complementary SSR marker analysis using three primer sets, AHF1+AHR1, AHF2+AHR2, and AHF3+AHR3, to amplify DNA. Optimal amplification temperatures for the PCR reactions ranged from 55 to 58°C, and clear bands were observed at 150 and 250bp.

The band patterns were consistent across all lines, signifying the high similarity in their genetics, which relayed their relatively low genetic distance among the samples. Despite the known polymorphism of SSR markers in some cases, the diversity observed in this study would indicate a genetic uniformity within these lines produced from a few products of original crosses. Optimal amplification temperatures fall between 55°C and 58°C, per existing protocols for the processing of SSR amplification, which usually demands a narrowly defined temperature to help with the specificity and efficiency of amplification (Khaing et al. 2013; Oo and Park, 2013). The genetic uniformity observed among the selected lines could be due to shared ancestry of the selected lines, or to limited variation within these lines. Other molecular markers such as RAPD may be a better means to capture more subtle genetic differences. Although SSR markers worked well to show similarities, an extension of the analysis to include other markers and larger populations may provide a clearer picture of the genetic variability in Amaranthus. Strong band pattern consistency among the 4 lines indicated that the samples were genetically uniform. This is in agreement with other studies that reported low genetic diversity levels for some Amaranthus accessions, in particular regions where breeding practice has favored uniformity over diversity (He and Park, 2013; Wang and Park, 2013). For example, U.S amaranth accessions showed low genetic diversity due to historical breeding in this region (He and Park, 2013). It also emphasizes the low degree of genetic exchange among Amaranth species in South and Southeast Asia and supplies the evidence of genetic uniformity within a particular accession (Wang and Park 2013). Minimal genetic distance among the lines poses a problem to efforts aimed at creating hybrids to have traits needed that are important in the adaptation of Amaranthus to changing environmental conditions, including stress tolerance and nutritional quality (Jamalluddin et al. 2022; Singh et al. 2023).

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5. CONCLUSION

This study demonstrated the potential of in vitro techniques for improving Amaranthus crop regeneration and genetic evaluation. Optimal sucrose concentration (30g/L) and hormonal combinations effectively enhanced seedling growth and callus formation. The A-20 and A-21 lines showed strong responses to 2,4-D and kinetin, while callus regeneration succeeded with NAA and kinetin or Zeatin and IAA. SSR marker analysis revealed genetic similarity among the lines, indicating low variability. This highlights the need to expand genetic resources in Amaranthus breeding programs to improve stress tolerance and adaptability. Overall, these findings provide a foundation for enhancing Amaranthus propagation and breeding strategies.

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Research involved human and animal rights: This research did not involve experiments with human or animal participants.

Conflicts of Interest: The authors declare no conflict of interest.

Author's Contribution: Muhammad Ahmad and Saba Sadiq performed the main research work, and other authors helped with data compiling, statistical analysis, and paper writing in this research work.

Data Availability: All data generated or analyzed during this study are included in this article.

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