RESEARCH ARTICLE

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Pioneering anther culture-based embryogenesis in *Solanum aethiopicum* L.

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Abstract
This study aimed to establish a compelling approach for inducing embryogenesis through *in vitro* anther culture in Scarlet eggplant (*Solanum aethiopicum* L.), the most consumed and popular eggplant among indigenous vegetables on the African continent. While *in vitro* androgenesis has been favorably employed in brinjal (*Solanum melongena* L.) breeding, there has been no attempt to induce embryogenesis in a large germplasm of its relative, *Solanum aethiopicum*. In two distinct experiments, the largest germplasm collection of *Solanum aethiopicum* gr. Gilo was assessed for embryogenesis induction using C medium supplemented with different concentrations of hormones. In the first experiment, callus induction was successful with an overall rate of 36.6 calli/100 anthers, but embryo formation was unsuccessful. Statistical analysis revealed a dependency of the rate of callus induction on accessions. In the second experiment, only four selected accessions of *Solanum aethiopicum* gr. Gilo were used and compared to two Turkish eggplant genotypes of *Solanum melongena* in two distinct treatments. The results showed that in the first treatment (I), only the accession GKE12 had a satisfactory outcome with a rate of embryo formation of 0.82/100 anthers and 0.41/100 anthers corresponding to the rate of developed embryos. In the second treatment (II), only controls, which were Adana and Kemer cultivars of *Solanum melongena* formed embryos with a rate of 7.26/100 anthers and 1.15/100 anthers, respectively. The obtained embryo/seedling of *Solanum aethiopicum* gr. Gilo was found to be diploid. Overall, this study demonstrated that with the right combinations of hormones, it is possible to induce embryogenesis and produce a diploid of *Solanum aethiopicum*, the world's second most popular cultivated eggplant after brinjal. These findings could potentially contribute to the breeding of eggplants for enhanced genetic variation and resistance.
Keywords: African eggplant, Embryo formation, *In vitro* androgenesis, Microspore-derived embryos, Plant breeding

INTRODUCTION

In vitro anther culture is a useful technique for generating doubled haploid plants in economically variable crops such as eggplant. Indeed, haploid plants are very useful in breeding programs. They help with the detection of recessive mutations and the attainment of F1 hybrid vigor (Alpsoy and Şeniz, 2007). Anther culture is used in addition to conventional breeding methods to produce pure (homozygous) lines. They are produced through androgenesis by generating doubled haploid (DH) plants. Consequently, hybrid plants are produced by crossing two pure (homozygous) lines with desirable characteristics (Seguí-

Simarro, 2016).

The *in vitro* anther culture technique has been consistently used to induce the establishment of plants from microspores via direct and indirect embryogenesis. Since the 1980's, anther culture has been used in eggplant to produce double-haploid plants from microspore-derived embryos (Rotino, 2016). The generation of androgenic doubled haploid (DH) lines from haploid microspores/pollen represents an attractive option to conventional breeding techniques for the production of pure lines or 100% homozygous lines for hybrid seed production in high-value crops. It is well documented that DH technology is one of the most efficient and cost-effective methods for accelerating the development of pure lines from anther culture (Salas et al., 2012; Calabuig-Serna et al., 2020). DH technology is still a long way from becoming an omnipresent technique for producing pure lines on a regular basis. In some cases, DHs are obtained, confirming the recalcitrant character of eggplant species/cultivars. This technology is considered to be species-dependent. It is also affected by the microspore's developmental stage and other factors, such as the physical and chemical settings of the *in vitro* culture system (Salas et al., 2012).

This technique has been optimized and extensively used for more than four decades, for commercial and experimental purposes, by which accelerated generation double-haploid parental lines of F1 hybrids are achieved. Furthermore, microspore-derived plants facilitate genetic analysis due to their complete homozygosity characteristics (Rotino, 2016). A handful of studies have been conducted to improve eggplant (*Solanum melongena* L.) through anther culture (Khatun et al., 2006). Other researchers like Kumar et al. (2003), Alpsoy and Şeniz (2007), Salas et al. (2011), and Başay and Ellialtıoğlu (2013) have all successfully applied anther culture to *Solanum melongena*. Salas et al. (2011) evaluated androgenesis induction through anther culture by comparing both common eggplant accessions to other related species, including one cultivated scarlet eggplant (*Solanum aethiopicum*) accession that produced 21.5 calli/100 anthers while no embryo was observed.

Sources indicate that determining the optimal development stage of microspore/pollen for successful androgenesis in different eggplant genotypes, particularly *Solanum melongena*, is more challenging. Thus, in most cases, visual references or morphological criteria differ between cultivars, or even between buds from the same plant donor. However, it has been reported that the proper growth phase from a morphological standpoint should have sepals and petals equal or petals 1-2 mm higher than sepals (Vural et al., 2019). Consequently, Salas et al. (2012) disclosed that younger anthers with mostly young and mid-microspores are preferable for anther culture. Mir et al. (2021) claim that at this younger stage, microspores are near the beginning of pollen mitosis, allowing embryogenesis to be induced more appropriately. However, this condition is not always met because all microspores in an anther are not always at the same stage. Different stages, on the other hand, exist side by side within the same anther. Younger anthers are also preferred because their walls are less thick, allowing media components and growth factors to diffuse and attain microspores inside the locules during *in vitro* anther culture.

In summary, considerable media formulations and inductive treatments have been employed to generate double haploids through anther culture in varied eggplant F1 hybrids and cultivars in alternative experimental scenarios (Başay and Ellialtıoğlu, 2013). The practical usefulness of anther culture in comparison to other androgenesis induction techniques, such as isolated microspore culture, has made it one of the most widely used techniques in eggplant, primarily in cultivated eggplant (*Solanum melongena* L.), with the target of acquiring double haploid parents for conventional breeding (Kashyap et al., 2003; Vural and Ari, 2020). To the best of our knowledge, there has been little research into androgenesis induction in *Solanum melongena* relatives. This is the primary reason why scarlet eggplant (*Solanum aethiopicum* L.), another cultivated eggplant that has long been neglected in scientific research (Shimira et al., 2021), was chosen for determining anther culture's performance in this study. As a result, embryogenesis was attempted on the largest germplasm collection made up of several accessions of *Solanum aethiopicum* gr. Gilo in this study.

The objectives of this research were to uncover the androgenic capacity of African eggplant using a germplasm collection of *Solanum aethiopicum* landraces originated from Rwanda, as well as the accessions/landraces' effects on the ability to induce haploid embryos and convert them to embryo-derived plantlets.

MATERIALS AND METHODS

Plant materials and growing seasons

Plant materials used for the in vitro anther culture study were from the collection of *Solanum aethiopicum* gr. Gilo originated from Rwanda (Shimira et al., 2021) and were maintained in a greenhouse located at the horticultural research application area of Cukurova University (37°01'46.1"N 35°22'02.7"E). For the purpose of this experiment on embryogenesis induction, there were two growing seasons. The first growing season in 2020 lasted from June to December. The second growing season in 2021 lasted from May to November. Well-developed eggplant plantlets were moved from plant growing trays to an experimental field bed for the study. The plants were planted in a randomized block configuration with eight rows and three replications of each accession spaced apart by 1.4 meters and 0.75 meters, respectively. Water-soluble nutrients were applied to the plants, and

drip irrigation was employed to keep the soil moist. Weeds were removed once a week, and other agronomic practices, such as pruning and chemical application, were carried out to manage pests and diseases.

For the first growing season, the whole eggplant germplasm collection of *Solanum aethiopicum* gr. Gilo made up of 60 different accessions was grown. Unfortunately, due to the quality of the seeds only 52 accessions were able to grow and provide enough flower buds for *in vitro* anther culture. Meanwhile, in the second growing season, fewer accessions of *Solanum aethiopicum* gr. Gilo (GKE 12, GKE20, MZE24 and MZE53) were grown with two local cultivars of *Solanum melongena* (Adana and Kemer).

Samples collection

An *in vitro* anther culture experiment was carried out at the Prof. Dr. Saadet BÜYÜKALACA - Tissue Culture Laboratory (Horticulture Department, Çukurova University) in Adana, Türkiye, from October 2020 to January 2021 (first experiment), then from September 2021 to March 2022 (second experiment) to evaluate androgenesis capacity and embryo formation of scarlet eggplant (*Solanum aethiopicum* gr. Gilo) accessions chosen for their genetic diversity.

Flower buds were collected from donor plants in the greenhouse according to the three stages established by cytological examination (See below). In the most cases, the collected flower buds were in stages 1 and 2. A few samples were collected every morning and immediately transported into appropriate containers, as shown in Figure 1.

Figure 1. Eggplants' flower buds

(A) Flower buds in the greenhouse just before collection,

(B) Flower buds in the containers after collection

Control of suitable flower bud sizes and the development stage of microspores/pollen

The optimal development stage of microspores/pollen and also the ideal size of flower buds were evaluated using microscope observations. According to Vural et al. (2019), the best stage for microspores is when they are uninucleate before the beginning of initial pollen mitosis or binucleate at the start of cytokinesis. These observations permitted the connection between floral bud sizes and microspore developmental stages to be identified (Figure 2).

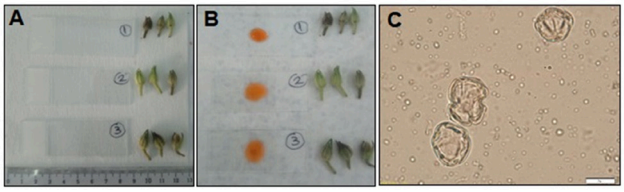

Figure 2. Flower bud size selection

(A) Flower bud types based on size, (B) Microspore staining with acetocarmine on labeled slides,

(C) Microspores observation under a microscope (Magnification 100X ~ 10 µm)

A few samples of freshly collected flower buds of *Solanum aethiopicum* gr. Gilo were used. Firstly, individual anthers were dissected out from flower buds and then crushed/squashed on petri dishes using a scalpel to expose micropores. Afterward, those microspores were stained with acetocarmine. The obtained mix was put on microscope slides and covered with glass slides for further cytological observation. From the cytological examinations, three suitable development stages for anther culture were established.

Media preparation

The protocol for media preparation used in this study was first proposed by Dumas de Vaulx et al. (1982) for anther culture in eggplants. Three distinct nutrient media (C, R, and V3) containing different plant growth regulators at various concentrations were used (Table 1).

The anther culture initiation is performed on C medium for the first 13 days. Then, R medium is used for sub-culturing untill embryos are obtained. Lastly, V3 medium is used for embryo growth and development. Details on the different concentrations of plant growth regulators are given in Table 2.

Experimental design for in vitro embryogenesis

• Experiment 1: "Effects of S. aethiopicum accessions and culture media on in vitro androgenesis induction"

In this experiment, the entire germplasm collection of *Solanum aethiopicum* gr. Gilo made up of 52 different accessions originated from Rwanda was essayed on C medium supplemented with 2.4-D (5 mg/l) and kinetin (5 mg/l). The experiment was conducted using 26 replications for each accession. Here, one accession that did not reach same number of replications was excluded from the study.

• Experiment 2: "Effects of selected S. aethiopicum accessions, 2 Turkish eggplant (Solanum melongena) varieties and 2 hormanal treatments on in vitro androgenesis induction"

In this experiment, the 4 accession lines (GKE12, GKE20, MZE24, and MZE53) of *Solanum aethiopicum* gr. Gilo and 2 local eggplant (*Solanum melongena*) cultivars (Adana

Table 1. Details on C, R and V3 nutrient media (mg/L)

	C medium	R medium	V3 medium		C medium	R medium	V3 medium
Macro nutrients				**Vitamin and amino acids**			
KNO_3	2150	2150	1900	Myo-inositol	100.00	100.00	100.00
NH_4NO_3	1238	1238	1650	Pyrodoxin HCl	5.500	5.500	5.500
$MgSO_4$-$7H_2O$	412	412	370	Nicotinic acid	0.700	0.700	0.700
$CaCl_2$-$2H_2O$	313	313	440	Thyamine HCl	0.600	0.600	0.600
KH_2PO_4	142	142	170	Calcium panthotenate	0.500	0.500	0.500
$Ca(NO_3)_2$-$4H_2O$	50	50	-	Vitamin B_{12}	0.030	-	-
NaH_2PO_4-H_2O	38	38	-	Biotin	0.005	0.005	0.005
$(NH_4)_2SO_4$	34	34	-	Glycin	0.100	0.100	0.200
KCl	7	7	-				
Micro nutrients				**Chelated Irons**			
$MnSO_4$-H_2O	22.130	20.130	0.076	Na_2-EDTA	18.65	18.65	37.30
$ZnSO_4$-$7H_2O$	3.625	3.225	1.000	$FeSO_4$-$7H_2O$	13.90	13.90	27.80
H_3BO_3	3.150	1.550	1.000				
KI	0.695	0.330	0.010				
Na_2MoO_4-$2H_2O$	0.188	0.138	-				
$CuSO_4$-$5H_2O$	0.016	0.011	0.030				
$CoCl_2$-$6H_2O$	0.016	0.011	-				
$AlCl_3$-$6H_2O$	-	-	0.050				
$NiCl_2$-$6H_2O$	-	-	0.050				

Table 2. Plant growth regulators and their concentrations

Growing Medium	Plant growth regulators		Sucrose	Agar
	2.4-D	Kinetin		
C medium	5*	5*	100**	8**
	1*	1*	100**	8**
R medium	0.01	-	30**	8**
V3 medium	-	-	30**	8**

*: mg/L, **: g/L

and Kemer) were tested on two distinct treatments and evaluated for *in vitro* endrogenesis induction on C medium. The experiment was conducted utilizing 31 replications for each accession/genotype. For treatment 1, C medium was supplemented with 2.4-D (5 mg/l) and kinetin (5 mg/l) while, for treatment 2, a 1 mg/l of 2.4-D and 1 mg/l of kinetin were supplemented in C medium.

Initiation of Anther Culture

For the disinfection step, a 20% sodium hypochlorite (NaOCl) solution was used for 15 minutes, succeeding four rinses with sterile distilled water (Figure 3).

By working under a sterile bench (laminar flow hood), the aseptic condition was maintained throughout the procedure of Başay and Ellialtıoğlu (2013). After dissecting the flower buds and carefully removing their filaments, anthers were carefully placed on the appropriate media to circumvent anthers dropping under the media surface. Petri dishes are sealed before being placed into the incubator (Shimira et al., 2019). Figure 4 illustrates the whole process of *in vitro* anther culture.

Excised anthers were cultured in the induction medium (C), complemented by proper plant growth regulators (Table 2), and installed at 35 °C in an incubator under darkness for 8 days, according to the protocol developed by Dumas de Vaulx et al. (1982). Figure 5 shows newly cultivated peti dishes in the incubator. Afterwards, petri dishes were kept in the growth chamber at 25 °C for 16 hours under fluorescent light (50 mol/m-2.s-1). Anthers were transferred to the differentiation medium on the 13th day (R). Sub-culturing can begin once embryos appear in V3 medium (Salas et al., 2012). In this study, R medium was renewed every 30 days while waiting for embryos development.

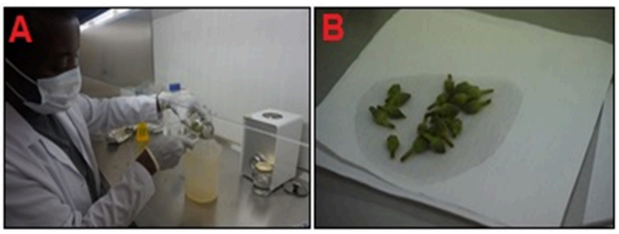

Figure 3. Sterilization process

(A) Flower buds sterilization under the bench,

(B) Sterilized flower buds on sterile tissue paper

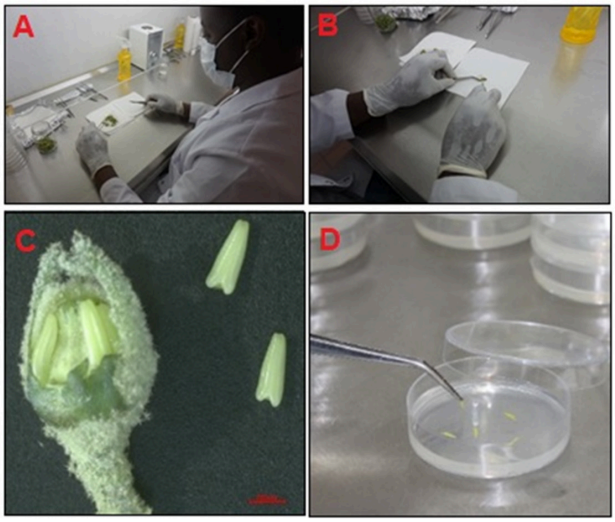

Figure 4. Anther culture process

(A) (B) (C) Flower bud and anthers, (D) Anther placement on media

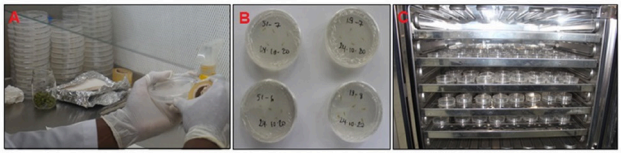

Figure 5. Major steps after anther culture

(A) The sealing of petri dish, (B) Sealed petri dishes ready to be incubated,

(C) Incubator containing newly cultured anthers

Embryo growth and development

The monitoring and observation of changes and callus development as well as embryo development were carried out by using a stereo microscope, Olympus SZ61 (Olympus Corporation, Shinjuku, Tokyo, Japan). This microscope, with high quality optics, allowed the thorough following of anthers' transformation to callus (Figure 6) and eventually the observation of a few embryos (Figure 7).

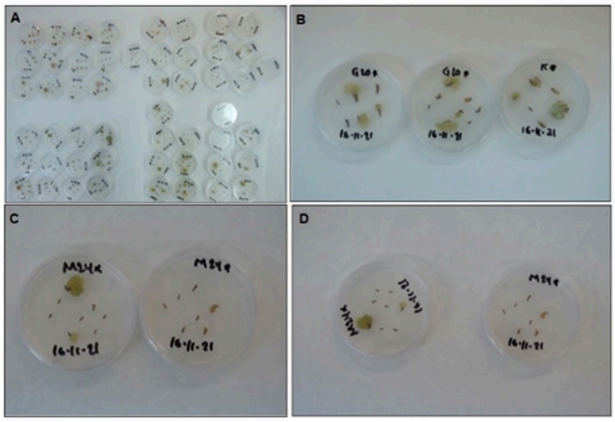

Figure 6. Petri dishes in the growing room (A), (B), (C) and (D) Petri dishes with some developed anthers

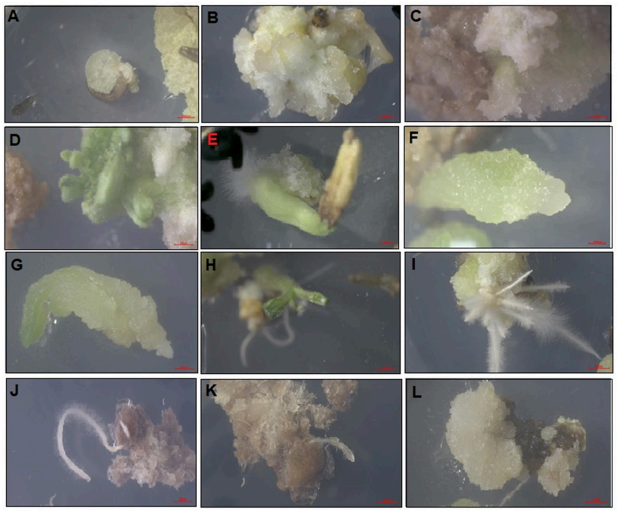

Figure 7. Embryo-like and embryo formations (A) to (L) Picture are shown at 300 µm

The protocol used by Vural and Ari (2020) for embryo conversion and acclimatization was slightly modified to meet the needs of our experiment. In brief, V3 medium was used after embryos were obtained to ensure their optimal growth and development. Firstly, embryos were transferred from petri dishes (R medium) to small glass flasks containing V3 medium (7 cm in height) (Figure 8A, Figure 8B, and Figure 8C). To obtain overall healthy embryos, grown embryos with slightly longer shoots and roots (or mature enough) were separated from their small siblings and transferred to new individual jars of superior width and height (7 cm, 8.5 cm, and 13.5 cm) containing V3 medium (Figure 8D).

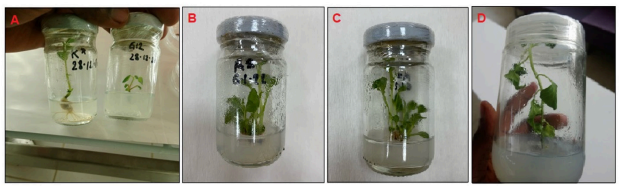

Figure 8. Eggplant seedlings into small jars

After forming strong shoots and roots, *in vitro* embryo-derived plantlets were transferred to pots with a mixture of peat moss and perlite (3:1 v/v) for acclimatization (Figure 9). They were later moved to a greenhouse and subjected to a gradually decreasing humidity and progressively rising illumination schedule.

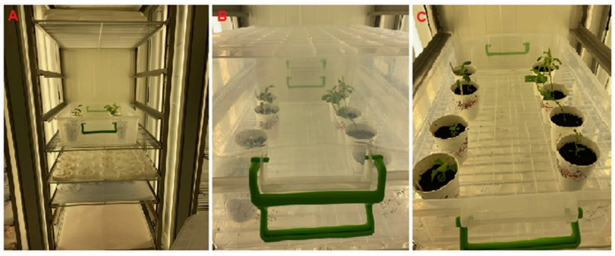

Figure 9. Embryo derived seedlings during acclimatization

Ploidy level analysis

Flow cytometry was employed to investigate the ploidy level. Fresh and young leaf samples from ex vivo embryo-derived plantlets (eggplant regenerants) were collected from the acclimation greenhouse and transported directly to the lab for ploidy determination. At this point, the research method used by Shimira et al. (2019) was employed. In that regard, leaf samples (0.5 cm^2 of leaf tissue per plantlet in individual petri dishes) were chopped with a harsh razor blade after adding 400 µl of extraction buffer (Figure 10). Afterwards, all these samples were incubated for 30 to 60 seconds. Following that, samples underwent filtration using a specialized filter (Partec 50 m Cell Trics®), and 1.6 ml of staining buffer was added to the sample tube for a brief incubation of 30 to 60 seconds before taking readings on a flow cytometer (Figure 11).

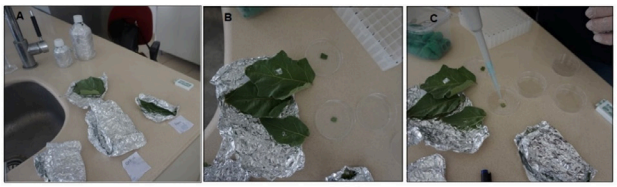

Figure 10. Collected leaf samples for flow cytometry analysis

Figure 11. Flow cytometry analysis

Data collection and analysis

The anthers were examined for callus initiation after four weeks of incubation. Furthermore, *in vitro* cultures were scored for the frequency of callus induction. The frequency was calculated as the ratio between the numbers of anthers responding to callus induction or regeneration to that of the total number of anthers inoculated as described by Kumar et al. (2003). For statistical analysis, descriptive statistics were conducted, and for advanced statistics, JMP (version 15.2.1, SAS Inc., Cary, NC, USA) statistics package software was used. An ANOVA test ($p \leq 0.05$) was conducted to evaluate general significant variations, and then a Fisher's least significant difference (LSD) test for multiple comparisons was used to sort different eggplant accessions into clusters, with significant differences at a p value < 0.05.

RESULTS AND DISCUSSION

Fist experiment

The numbers of cultured anthers and formed calli are described in Table 3. Unfortunately, since no embryos were obtained, only the frequency of callus formation was calculated in percentage per accession.

Results show that callus formation ranged between 55.90% and 20.23%, with the highest percentage for accession MZE34 and the lowest percentage for accession GKE8. The average callus formation percentage is 36.36 calli/100 anthers for the whole germplasm of *Solanum aethiopicum* gr. Gilo. For instance, the highest mean value of cultured anther was observed in GKE13 accession with values of 8.35±1.24 while the highest mean value of formed calli was noticed in MZE32 accession with 1.59±1.89.

The statistical analysis (one-way ANOVA) shows that accessions significantly influenced callus induction (Table 4). The statistical significance is shown by the small value of the P-value (compared to α=0.05). This can be taken as evidence that the means are different. Similarly, the mean comparisons through the least significant difference (LSD) test confirmed that the means were statistically different (Table 5).

Second experiment

For the second experiment, the number of cultured anthers as well as the number of formed calli and embryos are described in Table 6. Both the formation frequency of calli and embryos as well as the development of embryos were thoroughly calculated.

In the first treatment, results show that the GKE12 accession (*S. aethiopicum* gr. Gilo) was the only accession to have a good response regarding embryo formation and development, with 0.82 formed embryos per 100 anthers and 0.41 developed embryos per 100 anthers. Although, it was not the only accession to have formed embryos. Adana and Kemer eggplant (*Solanum melongena*) cultivars used here as control genotypes formed embryos with 0.61 formed embryos per 100 anthers and 1.19 formed embryos per 100 anthers, respectively. Briefly, the only developed embryo/

Table 3. Frequency of calli formation for the first experiment

N°	Accession ID	Petri Numbers	Number of Cultured Anthers	Frequency of Calli Formation	
				Number	%
1.	GKE1	26	186	72	38.71
2.	GKE2	26	190	46	24.21
3.	GKE5	26	177	88	49.72
4.	GKE7	26	201	71	35.32
5.	GKE8	26	173	35	20.23
6.	GKE9	26	175	54	30.86
7.	GKE11	26	202	74	36.63
8.	GKE12	26	207	68	32.85
9.	GKE13	26	217	58	26.73
10.	GKE14	26	157	85	54.14
11.	GKE15	26	196	72	36.73
12.	GKE16	26	182	44	24.18
13.	GKE17	26	180	57	31.67
14.	GKE18	26	182	59	32.42
15.	GKE19	26	180	78	43.33
16.	GKE20	26	200	77	38.50
17.	MZE22	26	204	96	47.06
18.	MZE23	26	183	85	46.45
19	MZE24	26	199	97	48.74
20.	MZE26	26	183	55	30.05
21.	MZE27	26	170	64	37.65
22.	MZE28	26	197	70	35.53
23.	MZE29	26	201	90	44.78
24.	MZE30	26	202	64	31.68
25.	MZE32	26	199	71	35.68
26.	MZE33	26	195	78	40.00
27.	MZE34	26	195	109	55.90
28.	MZE35	26	191	46	24.08
29.	MZE36	26	207	62	29.95
30.	MZE37	26	200	86	43.00
31.	MZE38	26	194	59	30.41
32.	MZE39	26	182	64	35.16
33.	MZE40	26	190	65	34.21
34.	MZE41	26	189	102	53.97
35.	MZE42	26	187	61	32.62
36.	MZE43	26	203	70	34.48
37.	MZE44	26	167	59	35.33
38.	MZE46	26	181	89	49.17
39.	MZE47	26	183	40	21.86
40.	MZE48	26	207	67	32.37
41.	MZE49	26	190	68	35.79
42.	MZE50	26	207	96	46.38
43.	MZE51	26	173	53	30.64
44.	MZE52	26	180	70	38.89
45.	MZE53	26	174	64	36.78
46.	MZE54	26	205	70	34.15
47.	MZE55	26	211	49	23.22
48.	MZE56	26	177	51	28.81
49.	MZE58	26	192	73	38.02
50.	MZE59	26	199	71	35.68
51.	MZE60	26	189	75	39.68

Table 4. Statistical anlysis on callus induction (First experiment)

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob > F**
Accessions	50	518.1568	10.3631	3.6274	<.0001
Error	1301	3716.846	2.8569		
C. Total	1351	4235.003			
Effect Tests					
Source	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Accessions	50	50	518.1568	3.6274	<.0001

seedling was attained from GKE12.

Regarding callus induction in the first treatment, there was a great variation within the 4 different accessions of *S. aethiopicum* gr. Gilo and the two control *Solanum melongena* cultivars (Adana and Kemer) as shown in Figure 12. For instance, the highest mean values of cultured anther and formed calli were observed in the MZE24 accession, with values of 2.97±1.91 and 3.23±1.58, respectively.

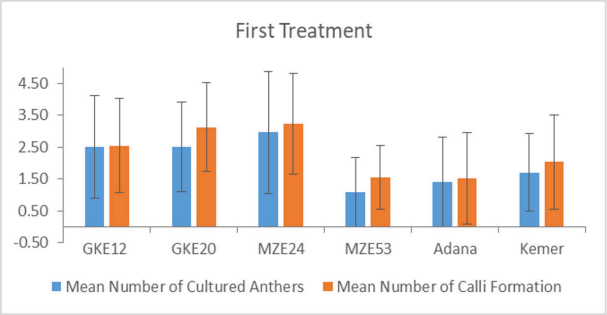

Figure 12. Callus Induction Details with Mean and SDs (Experiment II – First treatment)

In the second treatment, results show that none of the eggplant accessions (*S. aethiopicum* gr. Gilo) were able to form embryos. Embryo formation and development were observed in the two control *Solanum melongena* cultivars; Adana and Kemer, with 7.26 formed embryos per 100 anthers and 1.15 formed embryos per 100 anthers, respectively. For the frequency of developed embryos/seedlings, these values slightly decrease for Adana and Kemer, with 6.70 developed embryos per 100 anthers and 0.57 developed embryos per 100 anthers, respectively.

Regarding callus induction in the second treatment, there was a great variation within the 4 different accessions of *S. aethiopicum* gr. Gilo and the two control *Solanum melongena* cultivars (Adana and Kemer) as shown in Figure 13. For instance, the highest mean value of cultured anther was observed in the GKE20 accession with values of 2.35±1.26, while the ultimate mean value of formed calli was observed in the MZE24 accession with 2.97±1.43.

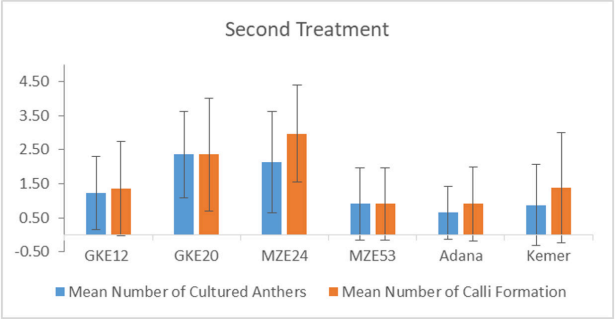

Figure 13. Callus Induction Details with Mean and SDs (Experiment II – Second treatment)

The statistical analysis (Two-way ANOVA) shows that accessions/genotypes and treatments (I and II) significantly influenced callus induction (Table 7). The statistical significance is shown by the small value of the P-value (compared to α=0.05). This can be taken as evidence that the means are different. Similarly, the mean comparisons through the least significant difference (LSD) test confirmed that means were statistically different (Table 8).

ANOVA results indicate that there was also a significant difference between treatments. That means that the factor "treatment" has an influence on the result obtained with regard to callus induction. At the accession/genotype level, we also found a significant difference. At the interaction level (Treatments*Accession/Genotype), we observed no significant difference.

Flow cytometric assessment of embryo-derived plantlets

Ploidy levels were measured in all obtained plantlets of *Solanum aethiopicum* gr. Gilo from the second experiment on *in vitro* androgenesis through anther culture. As shown in Table 9, 100% of all obtained plantlets were found to be diploid.

DISCUSSION

In the first experiment, the overall callus formation rate was 36.36 calli/100 anthers and there was no embryo formation. This value was greater than the value obtained by Salas et al. (2011), when they assessed androgenic capacity via anther in various genotypes of common eggplant and related species, including *Solanum aethiopicum*. They obtained 21.5 calli/100 anthers for the

Table 5. Details on LSD test (First experiment)

N°	Accessions	LSD results on the number of formed calli
1.	GKE01	2.77±1.53 EFGHIJK
2.	GKE02	1.77±1.21 MNOP
3.	GKE05	3.38±1.83 ABCDEF
4.	GKE07	2.73±1.64 EFGHIJK
5.	GKE08	1.35±1.41 P
6.	GKE09	2.08±1.57 JKLMNOP
7.	GKE11	2.85±1.93 CDEFGHIJK
8.	GKE12	2.62±1.68 EFGHIJKLM
9.	GKE13	2.23±1.66 IJKLMNOP
10.	GKE14	3.27±1.46 BCDEFGH
11.	GKE15	2.77±1.63 EFGHIJK
12.	GKE16	1.69±1.44 NOP
13.	GKE17	2.19±1.50 IJKLMNOP
14.	GKE18	2.27±1.80 IJKLMNO
15.	GKE19	3.00±1.92 CDEFGHI
16.	GKE20	2.96±1.95 CDEFGHIJ
17.	MZE22	3.69±1.78 ABCD
18.	MZE23	3.27±2.07 BCDEFGH
19	MZE24	3.73±1.59 ABC
20.	MZE26	2.12±1.53 IJKLMNOP
21.	MZE27	2.46±1.50 GHIJKLMN
22.	MZE28	2.69±1.93 EFGHIJKL
23.	MZE29	3.46±1.86 ABCDE
24.	MZE30	2.46±2.10 GHIJKLMN
25.	MZE32	2.73±1.93 EFGHIJK
26.	MZE33	3.00±1.72 CDEFGHI
27.	MZE34	4.19±1.60 A
28.	MZE35	1.77±1.63 MNOP
29.	MZE36	2.38±1.17 HIJKLMNO
30.	MZE37	3.31±1.76 ABCDEFG
31.	MZE38	2.27±1.80 IJKLMNO
32.	MZE39	2.46±1.45 GHIJKLMN
33.	MZE40	2.50±1.96 FGHIJKLMN
34.	MZE41	3.92±1.60 AB
35.	MZE42	2.35±1.72 IJKLMNO
36.	MZE43	2.69±1.67 EFGHIJKL
37.	MZE44	2.27±1.71 IJKLMNO
38.	MZE46	3.42±1.60 ABCDE
39.	MZE47	1.54±1.42 OP
40.	MZE48	2.58±1.65 EFGHIJKLMN
41.	MZE49	2.62±1.30 EFGHIJKLM
42.	MZE50	3.69±1.74 ABCD
43.	MZE51	2.04±1.15 KLMNOP
44.	MZE52	2.69±2.15 EFGHIJKL
45.	MZE53	2.46±1.27 GHIJKLMN
46.	MZE54	2.69±2.09 EFGHIJKL
47.	MZE55	1.92±1.71 LMNOP
48.	MZE56	1.96±1.46 LMNOPQ
49.	MZE58	2.81±1.88 DEFGHIJK
50.	MZE59	2.73±1.61 EFGHIJK
51.	MZE60	2.88±2.01 CDEFGHIJK

Table 6. Frequency of calli and embryos formation for the second experiment

Treatments	Access. IDs	Petri Numbers	Number of Cultured Anthers	Frequency of Calli Formation		Frequency of Embryo Formation		Frequency of Embryo Development	
				Number	%	Number	%	Number	%
I. (5 mg/l 2.4-D and 5 mg/l kinetin)	GKE12	31	244	78	31.97	2	0.82	1	0.41
	GKE20	31	243	78	32.10	0	0.00	0	0.00
	MZE24	31	234	92	39.32	0	0.00	0	0.00
	MZE53	31	200	34	17.00	0	0.00	0	0.00
	Adana	31	163	44	26.99	1	0.61	0	0.00
	Kemer	31	168	53	31.55	2	1.19	0	0.00
II. (1 mg/l 2.4-D and 1 mg/l Kinetin)	GKE12	31	213	38	17.84	0	0.00	0	0.00
	GKE20	31	231	73	31.60	0	0.00	0	0.00
	MZE24	31	234	66	28.21	0	0.00	0	0.00
	MZE53	31	204	28	13.73	0	0.00	0	0.00
	Adana	31	179	20	11.17	13	7.26	12	6.70
	Kemer	31	174	27	15.52	2	1.15	1	0.57

I: First treatment [C medium complemented with 2.4-D (5 mg/l) and kinetin (5 mg/l)].

II: Second treatment [C medium complemented with 2.4-D (1 mg/l) and Kinetin (1 mg/l)].

Table 7. Statistical analysis on callus induction (Second experiment)

Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Accessions/Genotypes	11	206.2876	18.7534	10.3803	<.0001
Error	360	650.3871	1.8066		
C. Total	371	856.6747			
Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatments	1	1	43.35753	23.9991	<.0001
Acc/Gen	5	5	148.4005	16.4284	<.0001
Treatments*Acc/Gen	5	5	14.52957	1.6085	0.157

Table 8. Details on LSD test (Second experiment)

	Level	Least Sq Mean		Level	Least Sq Mean
Accessions / Genotypes	GKE12	1.87 ^{B}	**Genotypes *Treatments**	I, GKE12	2.52 $^{A, B}$
	GKE20	2.44 ^{A}		I, GKE20	2.52 ^{A,B}
	MZE24	2.55 ^{A}		I, MZE24	2.97 ^{A}
	MZE53	1.00 ^{C}		I, MZE53	1.10 $^{D, E, F}$
	Adana	1.03 ^{C}		I, Adana	1.42 $^{D, E}$
	Kemer	1.29 ^{C}		I, Kemer	1.71 $^{C, D}$
Treatments	I	2.04 ^{A}		II, GKE12	1.23 $^{D, E, F}$
	II	1.35 ^{B}		II, GKE20	2.35 $^{A, B, C}$
				II, MZE24	2.13 $^{B, C}$
				II, MZE53	0.90 $^{E, F}$
				II, Adana	0.65 ^{F}
				II, Kemer	0.87 $^{E, F}$

Levels not connected by same letter are significantly different

Table 9. Ploidy levels in all *S. aethiopicum* gr. Gilo plantlets

Treatments	Accession IDs	No of embryo-derived plantlets	Haploid plantlets	Diploid plantlets
I (5 mg/l 2.4-D and 5 mg/l kinetin)	GKE12	1	-	1
	GKE20	-	-	-
	MZE24	-	-	-
	MZE53	-	-	-
	Adana	-	-	-
	Kemer	-	-	-
II (1 mg/l 2.4-D and 1 mg/l Kinetin)	GKE12	-	-	-
	GKE20	-	-	-
	MZE24	-	-	-
	MZE53	-	-	-
	Adana	12	-	12
	Kemer	1	-	1

I: First treatment [C medium with 2.4-D (5 mg/l) and kinetin (5 mg/l)].
II: Second treatment [C medium with 2.4-D (1 mg/l) and Kinetin (1 mg/l)].

sole genotype of *Solanum aethiopicum* utilized in their study, and it also failed to generate embryos. The results from ANOVA demonstrated the existence of a significant statistical difference among the entire germplasm collection of *Solanum aethiopicum* gr. Gilo with regards to callus induction.

For the second experiment (the first treatment), only one accession, GKE12 (*S. aethiopicum* gr. Gilo) formed embryos at a rate of 0.41 embryos per 100 anthers that gave plantlets. Controls made from Solanum melongena cultivars; Adana and Kemer demonstrated embryo production in both the first and second treatments, which is consistent with previous researchers' findings in anther culture studies of common eggplants. These researchers found embryo formation rates of 3,67 embryos/100 anthers in 'Kemer' (Alpsoy 2007), 14.2 embryos/100 anthers (Başay et al., 2011), 0.7–60.9 embryos /100 anthers (Salas, 2011) as well as 2.49–4.49/ embryos/100 anthers (Başay and Ellialtıoğlu, 2013).

Successful strategies for increasing the number of embryos and embryo-derived plantlets in *Solanum melongena* can be replicated in *Solanum aethiopicum*. For instance, Emrani Dehkehan et al. (2017) demonstrated that an individual supplement of 1 mg/l zeatin riboside and 10 mg/l mannitol in C medium (containing NAA and Kinetin) can positively affect anther culture outcomes. The highest embryo-derived plantlets obtained in this case were 25% and 66.6% for zeatin riboside and mannitol, respectively. Vural and Ari (2020) also demonstrated that a combination of activated charcoal, maltose, and silver nitrate in the original induction medium by Dumas de Vaulx et al. (1982) (DDV medium) had a triple synergistic effect on the high embryo yield of eggplant (*Solanum melongena* L.). And it can produce 3.9 times more embryos than the original DDV medium.

Obtaining doubled haploid plants in this type of eggplant, also known as the closest relative to brinjal, is a significant step forward for DH technology. This technology is reported to have the potential to speed up the process of generating new parental pure lines in several different species, including eggplant, which is considered a species that is moderately recalcitrant with regards to this technology. Eggplant breeding programs are primarily concerned with the release of hybrid varieties with better attributes. For instance, eggplant F1 hybrids, which are produced by crossing two parental homozygous plants, customarily outperform parental lines in terms of various agronomic traits (Mir et al., 2021).

CONCLUSION

This study demonstrated that embryogenesis induction through androgenesis can be successfully performed in one of the brinjal relatives, such as *Solanum aethiopicum* at a lower rate. *Solanum aethiopicum* accessions had significantly lower androgenic potential than *Solanum melongena* varieties. These findings highlight potential limitations for the widespread adoption of anther cultures in brinjal (*Solanum melongena* L.) relatives. Only the GKE12 accession responded and produced one embryo. The species was discovered to be the limiting factor in androgenic responses. The knowledge on androgenic responses gained in this study about local landraces of *Solanum aethiopicum* from Rwanda will provide a foundation for further research to develop doubled haploids to support ongoing efforts in the common eggplant (brinjal) breeding program as well as other breeding efforts in other cultivated eggplants.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and

that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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