Hungarian University of Agriculture and Life Sciences

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Improvement of somatic embryogenesis and androgenesis systems for sorghum [*Sorghum bicolor* (L.) Moench

Ph.D. Thesis

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Chege Paul Karumba

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Name of Doctoral School: Doctoral School of Plant Science

Discipline: Cereal Breeding and Biotechnology

Head of Doctoral School: **Prof. Dr. Lajos HELYES, DSc**
Hungarian University of Agriculture and Life Sciences
Director of Institute
Horticulture Research Group

Supervisor(s): **Prof. Erzsébet Kiss, PhD, CSc**
Hungarian University of Agriculture and Life Sciences
Institute of Genetics and Biotechnology

Prof. János Pauk, PhD, CSc, DSc
Cereal Research Non-profit Ltd
Department of Biotechnology

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Approval of Head of Doctoral School

……..

Approval of Supervisor(s)

1. INTRODUCTION

Sorghum is the fifth of the world's most consumed grains, a very important staple cereal crop to diets in the semi-arid tropics, where droughts cause frequent failures of other crops, and an important dietary alternative in northern Europe where there is prevalence of celiac disease, by providing safe and tolerable protein digests. It contributes to the food security of many of the world's poorest, most food-insecure agro-ecological zones, particularly in sub-Saharan Africa. The crop, together with other crops such as millet, peanuts and cowpeas is of particular interest in this region, because of its drought resistance, bearing in mind that 25% of the sub-Saharan Africa population lives in semi-arid regions. The farming in these drought areas has been mainly a subsistence activity with farmers producing a wide array of crops (including multiple cultivars of the same crop) for their own consumption, using few purchased inputs.

The harvested area of sorghum has increased, especially in Africa, although yields averaging 800 kg/ha remained unchanged. For this reason, various institutions including National Agricultural Research Systems (NARS), International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and International Sorghum and Millet Collaborative Research Support Program (INTSORMIL) have their breeding objectives oriented towards the yield reducing factors such as genetically low yielding landraces, drought, *Striga* weed, pests and diseases.

The delivery of these improved varieties needs to be hastened, in view of the ever-increasing demand for food due to increasing population, climate change effects that limit crop yields, increasing demand for feed and environment friendly industrial products such as bioethanol. This, however, has not been realized with many reviews indicating dismal release of improved cultivars. This trend has been a consequence of the lengthy period in classical breeding.

The biotechnology tool of doubled haploid technology may be tapped to reduce this lengthy period. The interest of breeders in haploids is that they lead to time saving in the production of inbred lines, they enable the production of strictly homozygous double haploids and unshifted screen i.e., double haploids will be the same in all the progenies.

DH technology has been used alongside Marker Assisted Selection (MAS), to speed up the development of some crops' traits such as disease resistance. These crops include wheat, barley, rice, cabbage, and maize. DH technology has shortened the period of development of these crops

from 8 -10 years to only two seasons. Even with the immense and urgent need to accelerate sorghum hybrid development, it is conspicuously missing out on these lists and the cumulative benefits thereof.

Many theories have been advanced for the low level of development of doubled haploid technology and generally the tissue culture of sorghum. To begin with, sorghum has been described as one of the most recalcitrant crops for tissue cultures. Several other factors that have significantly contributed to this slow development include low rate of callus induction and regeneration, high level of polyphenols exudation from somatic tissues and appearance of albino plantlets among regenerants. Genotype dependency is one of the great hindrances to sorghum tissue culture and successful doubled haploid production. Lack of an agreed type of culture medium that is reproducible is also a contributing factor to the low level of development in the sorghum tissue cultures. It may be argued also that the major cereals have dedicated laboratories in the developed countries that support large scale production of haploids, unlike sorghum, whose production until recently was mostly only important in the developing countries, with limited resources.

Objectives

To characterize selected Hungarian and African sorghum genotypes with microsatellite markers and to apply SSR analysis for confirming haploidy (or dihaploidy/homozygosity) of the regenerants.

To improve, optimize and adapt a sorghum somatic embryogenesis protocol.

To regenerate haploids from gametophyte haploid culture and to produce genetically homozygous F_2 progenies of sorghum [*Sorghum bicolor* (L) Moench]either by spontaneous or induced diploidization from an established breeding program.

2. MATERIALS AND METHODS

2.1 Assessing the genetic diversity of selected Hungarian and East African genotypes

2.1.1 Plant material

A total of 31 sorghum [*Sorghum bicolor* (L.) Moench] genotypes were used in the study, 15 of which were East African sourced from International Crop Research Institute for Semi-Arid Tropics (ICRISAT-Nairobi) and 16 Hungarian sourced from Cereal Research Non-profit Ltd, Hungary. Five seeds of each genotype were germinated on sterile forest soil in plastic boxes at the Institute of Genetics and Biotechnology at the Hungarian University of Agriculture and Life Sciences, Gödöllő

2.1.2 Genomic DNA extraction

Total genomic DNA for the East African genotypes was extracted following Aqua Genomic ™ plant protocol according to MoBiTec GmbH 2012©, from fourteen-days old young fresh leaves of each genotype. A similar methodology using the Omega™ DNA extraction protocol was followed for the Hungarian genotypes. The Aqua Genomic™ kit was found to be a relatively cheaper, faster and easier protocol sorghum DNA extraction, although it produced low quantities of genomic DNA, of relatively low purity as compared to the Omega™ kit.

2.1.3 Quality and concentration determination of genomic DNA

The genomic DNA was confirmed by running it on 1.0% agarose gel stained with ethidium bromide and visualized under UV light. The quality and concentration determination of the DNA was done using a Thermo Scientific Nano Drop ™ 1000 spectrophotometer. Stock DNA was diluted to a final volume of 50 ng/µl after normalization to 10 ng/µl.

2.1.4 SSRs markers and genomic DNA amplification using PCR

Out of 41 SSR markers used by (Billot et al., 2013) only five primers pairs that were highly polymorphic with a high allele number and at different chromosome numbers were selected. The forward primers were labelled with fluorescent dye (CY5) while the reverse primers were unlabelled. PCR amplification reactions were done in 10 µl reactions mixtures, containing 2 µl of diluted (10 ng/µl) template DNA, 0.75 µl of 10 µM forward primer which was labelled with fluorescent dye, and 0.75 µl of 10 µM reverse primers ,0.5 µl of 25 mM $MgCl_2$, 0.15 µl of 2 mM dNTPs, 0.3 µl Dream Taq™ polymerase (conc. 5 units/ml), 1.25 µl of 10x Dream Taq buffer, 4.3 µl sterile water. Bio Rad™ 100 and Gene Amp™ PCR system 9700 thermal cyclers were used to conduct PCRs, Touch down PCR protocol. It consisted of an initial denaturation step at 95°C for

2 minutes; 10 cycles comprising of denaturation for 30 seconds at 94℃, annealing for 30 seconds at 65℃, with a decrease of 1℃ in every subsequent cycle and an extension for 1 minute at 72℃; 24 cycles comprising of denaturation at 94℃ for 30 seconds, 55-56℃ annealing for 30 seconds, 72℃ for 1 minute for primer elongation; and then a final extension at 72℃ for 5 minutes. The amplified products were held at 4℃ and later stored at -20℃ for use onwards.

2.1.5 Electrophoresis of PCR products on agarose gel

The PCR amplicon size determination was done initially by running them on a gel-electrophoresis. On 1.0% agarose gel, 2 µl of the molecular DNA ladder was loaded on first well, while the other wells were loaded with 5 µl PCR products mixed with 2 µl loading dye. Electrophoresis was carried out at 110 V for 15 minutes. The gel after electrophoresis was visualized under on a UV transilluminator and the gel images recorded. The reproducibility of amplification products was confirmed for each primer by repeating the PCR and subsequently gel runs.

2.1.6 Detection of the PCR products on ALF Express Fragment II Analyzer™ machine

Two types of standards: external standard consisting of 70 bp ,95 bp, 150 bp, 275 bp and 300 bp size fragments of 7.5 µl from each and 262.5 µl sterile deionized water to a final volume of 300 µl serving as the molecular weight markers; and internal standard comprising of 70 bp, 95 bp and 300 bp size fragments determined by the size of the sample were utilized. 7.5 µl from each fragment size, 85 µl diluted cresol red dye and 200 µl sterile deionized water to a final volume of 300 µl were used to make the internal standard. Cresol red dye helped in visibility when loading the samples. 5 µl of the external standard mixed with 5 µl of sterile deionized water was loaded into wells 1, 20 and 40 of the gel, while a mix of 3 µl internal standard, 4 µl sterile deionized water and 2 µl of each PCR product were pipetted into the rest gel wells. All the samples were denatured at 94℃ to separate the dsDNA molecules into ss DNA molecules using Bio RAD T 100™ Thermal cycler for 5 minutes and placed on ice immediately before loading into the wells of the gel in the ALFExpress II Fragment Analyzer™ machine.

The samples were run for 50 minutes at 850 v,75 mA, 65 W at constant temperature of 42℃. On completion of the 1st run the wells were washed with the buffer and a repeat of the samples were loaded for the 2nd run and the same process repeated for 3rd run. The peaks of the external and internal standards were used to determine the correct allele sizes of the microsatellite loci.

2.1.7 Marker polymorphism and allele frequency

To measure the informativeness of the markers, the polymorphism information content (PIC) and allelic frequency for each genomic SSR marker was obtained by analyzing the scored data using MS Tool kit software. For the cluster analysis, the data was converted into binary codes and dendrogram constructed with SPSS version 23.

2.2 Suitability of 2,4-D and TDZ for somatic embryogenesis of sorghum

2.2.1 Plant material

Donor stalks were collected from 10 nursery grown sorghum genotypes. The donor genotypes including hybrids 'Alföldi 1', 'GK Emese', 'GK Zsófia', 'Farmsugro 180', 'Róna 1', 'GK Áron' and candidates 'V4', 'V5', 'V6' and 'V7' were selected from the sorghum hybrid breeding program of Cereal Research Non-profit Ltd., Szeged, Hungary.

2.2.2 Sterilization

The unopened stalk segments were cut in the laboratory to about 8 cm towards the end containing the floral meristem. The stalk segments that included the young inflorescence were sterilized in 50-50% solution of bleach (8% sodium hypochlorite) and sterile distilled water plus a drop of Tween 20 with agitation for 20 minutes. This was followed by rinsing three times in sterile distilled water (Millipore Elix 5). The sterilized stalk segments were then placed upright in sterilized and capped glass bottles under a laminar air flow cabinet.

2.2.3 Assessment of the hormones

The stalks were then longitudinally cut in the laminar air flow cabinet to reach the young inflorescence meristem. The buds were then cut into small pieces (1 mm). Ten of the pieces from a common explant were placed on each 90×15 mm Petri dishes containing induction medium, half strength MS medium (Murashige and Skoog, 1962) supplemented with 2 mgl-1 2,4-D, 2 mgl-1 TDZ, 5 mgl-1 2,4-D and 5 mgl-1 TDZ labelled as T1, T2, T3 and T4 respectively. The Petri dishes were incubated in the dark thermostat at 28°C for 5 weeks and the number of the type of calluses formed out of the 10 in each Petri dish and their colour was recorded each week.

2.2.4 Experimental design and data analysis

A Randomized Complete Block Design (RCBD) was used with 10 genotypes and 4 media (10 × 4 = 40 treatments). For each genotype, the treatments consisted of 4 replications of each medium, each replication with 10 pieces of the young inflorescence explant. A percentage of the counts of each callus type and their colour was determined for each replication every week for five weeks.

The differences of means for the various callus types and colours in the four treatments was obtained by Kruskal Wallis test in R Commander X64 3.4.4 edition software. The means of treatments with the 10 varieties were compared by Duncan's Multiple Range Test using RStudio software. Plots showing the trends in development of the different callus types and differences in means for the callus types over the collection phases were done using RStudio software.

2.2.5 Regeneration of calli and rooting media

Embryogenic calli from 3 of the 10 genotypes under study namely 'GK Emese', 'GK Zsófia' and 'Róna 1'were incubated under light in regeneration media denoted R1, R2 and R3 respectively in 90×15 mm plastic Petri dishes (Sarstedt, Newton, MA, USA). This was done 4 weeks after induction. The regenerated shoots were subsequently transferred to 190-2 medium in glass and incubated under light for rooting induction. Data of the regenerants in the three-regeneration media was analyzed with R Commander X64 3.4.4 edition software.

2.3 Optimizing tissue culture media for sorghum somatic embryogenesis

2.3.1 Plant material

Ten genotypes namely hybrids 'Alföldi 1', 'GK Emese', 'GK Zsófia', 'Róna 1', 'GK Áron' ,'GK Erick', 'GK Csaba', and candidates 'ARET×VSZ21KKD', '(A119×KS60B)×SMRIL', 'AIL-1× B119×Va-Cir' as varieties V01, V02, V03, V09, V10, V12, V16, V05, V14 and V15 respectively were obtained from a breeding program at Cereal Research Non-Profit Ltd., Szeged, Hungary.

2.3.2 Sterilization of plant explants

A set of 100 mature seeds from each genotype was sterilized separately by soaking them in 70% ethanol for 2 minutes and followed by agitation in 4% sodium hypochlorite and two drops of tween (in 100 ml) 20 for 30 minutes. This was followed by 3 washes with sterilized distilled water in a laminar air flow cabinet. The seeds were dried on sterile filter papers before placing them on a half strength MS (Murashige and Skoog, 1962) basic medium for germination in light.

2.3.3 Comparison of the induction media

To test the efficacy of the six medium types (Tab. 1) in callus induction, twenty 1 mm pieces of mesocotyls from 1.5-day old germinating embryos were placed on each media type contained in 90×15 mm plastic Petri dishes (Sarstedt, Newton, MA, USA). Each Petri dish was a treatment in the Randomized Complete Block Design (RCBD) experiment, comprising of 3 replications. All the medium types were autoclaved at 121˚C for 20 mins after appropriate pH adjustment. The explants were incubated for four weeks in a dark thermostat at 28˚C.

Counts of the yellow and brown calli were recorded as a percentage of the total explants in the Petri dish. The same procedure was applied for all the friable yellow coloured calli.

2.3.4 Statistical analyses

Means of yellow, brown and friable calli with the six treatments and 10 varieties were calculated and compared by Duncan's Multiple Range Test and two-way Analysis of Variance (ANOVA) using RStudio and R software edition x64 3.4.4 software. Plots showing the means of yellow, brown and friable calli as well as those depicting differences in the means with the six treatments were made using RStudio software.

Table 1. The callus induction medium types under trial denoted with letters A-F

Medium type	Constituents
A	½ MS -2.2 g/l MS powder (MS0222), 2mg/l 2,4-D, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.7
B	½ 190-2 (Zhuang and Xu, 1983), 2 mg/L 2,4-D, 20 g/l sucrose, 7 g/l agar, pH 5.8
C	½ N6 (Chu et al. 1975) with 950 mg/l KNO_3 and 825 mg/l NH_4NO_3, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.8
D	½ B5 (Gamborg et al. 1968) powder (1.58 g), 2 mg/l 2,4-D, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.8
E	A (½ MS -2.2 g/l MS powder (MS0222), 2 mg/l 2,4-D, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.7), 1.0 g/l KH_2PO_4, (1.0 g/l L-proline, 1.0 g/l L-asparagine and 0.16 mg/l $CuSO_4 \cdot 5H_2O$) - filter sterilized
F- Control Liu et al. 2015	MS- 4.4 g/l MS powder (MS0222), 2 mg/l 2,4-D, 20g/l sucrose, 2.8 g/l Gelrite, pH 5.7 1.0 g/l KH_2PO_4, (1.0 g/l L-proline, 1.0 g/l L-asparagine and 0.16 mg/l $CuSO_4 \cdot 5H_2O$) – filter sterilized

2.4 Doubled haploid production using an improved anther culture protocol for sorghum

2.4.1 Plant material

Over a period of 24 months i.e. summer of 2018, winter of 2018/2019, summer of 2019 and winter of 2019/2020, twenty-eight Hungarian sorghum genotypes, including F_1 registered- and experimental hybrids were planted in the field and glasshouses in summers and winters

respectively. The repeated plantings served as seasonal replications. All the sorghum genotypes were obtained from the sorghum breeding program of Cereal Research Nonprofit Ltd., Szeged, Hungary. Panicles were harvested while enclosed in the leaf sheath just before their lateral expansion caused the leaf sheath to split open, in the early vacuolated, uni-nucleated stage of microspores.

2.4.2 Pre-treatment and sterilization

Following confirmation of the right stage of the microspores (early vacuolated, uni-nucleated stage) using an Olympus CK-2 inverted microscope (Olympus, Southern-on-Sea, UK) at ×40 magnification, panicles from the summer of 2018 were placed at 4˚C for 10 days while those from the winter of the same year were placed at 4˚C, 8˚C and 10˚C each for 7, 10 and 15 days in lighted phytotron chamber at 85% relative humidity prior to disinfection. Following the results of year 2018, the panicles from the summer of 2019 and winter of 2019/2020 were held at 10 ˚C for 15 days prior to sterilization in the lighted phytotrons at 85% relative humidity. After their respective pre-treatment periods, panicles were removed from the leaf sheaths and disinfected by agitation in 100 ml of 4% sodium hypochlorite adding two drops of 'Tween 20' for 30 minutes. This was followed by 3 washes with sterilized distilled water in a laminar air flow cabinet.

For starvation pre-treatment trial, the isolated anthers from the summer of 2018, prior to incubation in 3 different induction media were placed in 3 replicates in 0.3 M mannitol while the controls were directly placed on the induction media, and both were placed at 32˚C in a thermostat for 3 days. The anthers (33 anthers/Petri dish) in the 0.3 M mannitol were then transferred to the induction media, following which all of them contained in 90 mm × 15 mm plastic Petri dishes (Sarstedt, Newton, MA, USA) were placed at 28˚C in dark thermostat. Following the results of the starvation trial in year 1, anthers from the summer and winter of 2019/2020 were not placed in 0.3 M mannitol but were all placed at 32˚C heat shock pre-treatment in a thermostat for 3 days before being transferred to the induction temperature of 28˚C in another thermostat.

2.4.3 Callus induction media

Three media including N_6 medium (Chu et al., 1975), MS-t-z-2 – a modified Murashige and Skoog medium (Murashige and Skoog, 1962) – and a Ficoll®400 supplemented W14mf medium as described by Puolimatka and Pauk (2000), were used in the calli induction for the anthers from the summer of 2018. Following the results of the summer of 2018, the callus induction media used for the anthers from the winter of the same year and summer of 2019 were: W14mf (M1) as a control,

W14mf supplemented with 1.0 g/l L-proline, 1.0 g/l L-asparagine and 1.0 g/l KH_2PO_4 (M2); and w14f supplemented with 1.0 g/l L-proline, 1.0 g/l L-asparagine but without KH_2PO_4 (M3). In the winter of 2019/2020, the induction media was M2, following results from the preceding summer's work.

2.4.4 Regeneration and hardening

The white to yellow embryogenic calli obtained from the anther culture on the induction media were regenerated on 190-2Cu medium (Pauk et al. 2003; Chege et al. 2020) under light condition at 25±˚C for 20-30 days. The regenerants obtained were severally cloned and sub-cultured on the same regeneration medium. The plantlets were then transferred to a glasshouse at 4 leaf stage into sand and soil (1:1) soil mixture and put under a PVC cover for 4 days after the transfer.

2.4.5 Ploidy-level determination and chromosome doubling

On attainment of the 4-leaf stage, all the regenerants' ploidy level was confirmed directly through flow cytometry. Diploid counterparts of the regenerants were included in the tests as control. A confirmatory test involving DNA extraction and PCR with select SSR markers (Billot et al. 2013) was also conducted for some of the regenerants by determining allele sizes following Polyacrylamide Gel Electrophoresis (PAGE) on ALFExpress II DNA fragment analyser machine.

Colchicine treatment was applied for two plants of 'Róna 1' regenerants from the induction work of the winter of 2018/2019 The colchicine treated 'Róna 1' plants were then planted alongside the other regenerants of the same variety to check the level of occurrence of spontaneous diploidization after transplantation and its effectiveness in comparison with colchicine mediated diploidization in sorghum. This comparison was performed based on the survival rate of the colchicine treated plants, and fertility levels of the panicles determined by the average number of filled grains of three randomly selected panicles from each of priory three panicle groups arranged based on visual observation of panicle fertility, as a percentage of the average number of the florets in those panicles.

Data on callus induction on the three modified W14mf media M1, M2 and M3 was analysed using Ri386 software version 3.6.2, where differences in the induction were analysed by a Kruskal-Wallis test. Histograms and boxplots depicting the differences on the callus induction were drawn using RStudio software.

3. RESULTS

3.1 **Assessing the genetic diversity of selected Hungarian and East African genotypes**

Following genomic DNA extraction, PCR with select SSR primers and eventual PAGE analysis, the correct allele sizes were obtained, which were useful in the dendrogram construction thus enabling the sorghum varieties to be clustered according to their genetic closeness. The two fragments (108:126 bp) of sample 24 corresponding to the variety Róna 1 clearly separated on NuSieve agarose gel. After the haploid induction, this locus together with the other heterozygous locus (mSbCIR238) of Róna 1 was applied for molecular proving of homozygosity, that is the success of haploid production.

The clustering of the genotypes was based on similarity matrix using an Unweighted Pair Group Method with Arithmetic average (UPGMA) algorithm to construct a dendrogram, which was obtained from binary data deduced from allele sizes of different markers used on the genotypes (Fig. 1). Genetically similar genotypes were clustered together. The cluster analysis resolved the 31 sorghum genotypes into two major clusters at 16% similarity threshold excluding genotype EC Teso which was dissimilar to the rest of the germplasm**.**

A total of 17 alleles were detected across the 31 sorghum genotypes by 5 polymorphic SSR markers in this study. The number of alleles generated per locus by each marker ranged between 2 for primers mSbCIR248 and mSbCIR262 and 6 for primer Xgap-206, which falls in the range of 2-9 alleles per SSR locus for various classes of microsatellites as reported in the work of Shehzad et al. (2009) using a different set of sorghum germplasm. The average number of alleles per locus detected was 3.4, which is relatively higher as compared to 3.2 in the work of Ali et al. (2008) and lower than 3.8 alleles per locus reported by Shehzad et al. (2009). Similarly, the average allele number was lower than that reported by Folkertsma et al. (2005) and Assar et al. (2005) where the average number of alleles per locus revealed was 7.3 and 6.1 in Guinea-race sorghum landraces and sorghum germplasms of Sudan, respectively. This indicates a lower genetic diversity in the present study material as compared to those in the works mentioned. Although Billot et al. (2013) recorded alleles in the range of 3-39, they reported that the broad number of genotypes in the trial (more than 3000 genotypes) was the reason for the high number of alleles per locus and other diversity parameters than for most of the other studies.

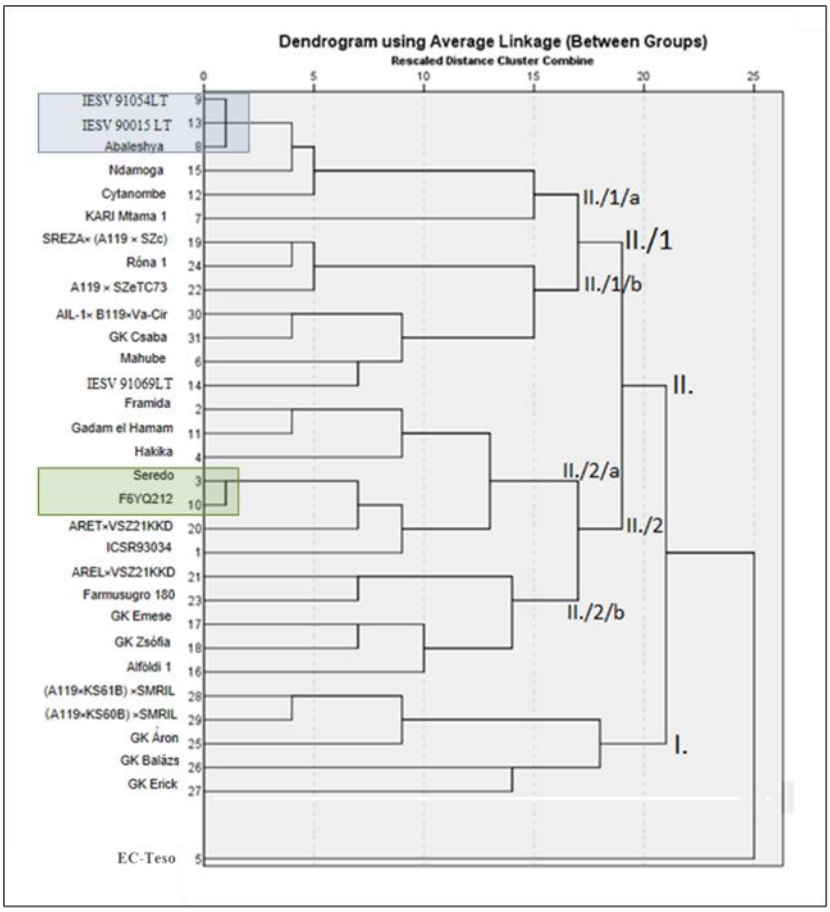

Figure 1. Dendrogram based on microsatellite analysis results of 31 Sorghum genotypes. (15 East African and 16 Hungarian genotypes).

In the present study, the observed heterozygosity (Ho) was generally lower than the expected heterozygosity (He) in all the loci apart from MsBCIR 262, which implied evenness of alleles with high homogeneity as expected since majority of the germplasms under study were hybrids. To measure the informativeness of each SSR marker, PIC value was calculated. In the present study, the PIC values varied widely among the SSR loci tested and ranged from 0.06 (msBCIR 262) to 0.615 (XGAP 206), with an average of 0.42 per locus. Marker MsBCIR 262 was the least informative with a PIC value of 0.06 while marker XGAP206 was the most informative with a PIC value of 0.615. For the genotypes in this study, the 5 markers obtained from the work of Billot et al. (2013) were not highly informative with an average PIC value of 0.421.

3.2 Suitability of 2,4-D and TDZ for somatic embryogenesis of sorghum

Four types of calli were observed in the cultures and were categorized as embryogenic calli (EC), embryogenic brown callI (EB), non-embryogenic soft (NES) and non-embryogenic brown (NEB).

EC at best quality to regenerate into plantlets were white in color, compact when touched with forceps, and contained nodule-like structures around them. The non-embryogenic structures were soft, watery and loose, and their colour was yellowish or brown. It was observed that some non-embryogenic structures would change to embryogenic although at very low levels. The results therefore major on the EC% means as influenced by the four media, genotype as well as the period under incubation.

Although hybrid 'Alföldi 1' had the highest EC% mean (46.0) at day 35, it was observed that all the EC% means were not significantly different ($P > 0.05$). 'Farmsugro 180', 'GK Emese', 'GK Zsófia, 'Róna 1' and candidates 'V4', 'V6' and 'V7' had higher EC% means on day 7 than on day 35, meaning some of the EC turned brown over the induction period. On the other hand, some non-embryogenic calli for hybrids 'Alföldi 1', 'GK Áron' and candidates 'V5' changed (Tab. 2) to embryogenic character, thereby making the EC% means at day 35 to be higher than at day 7.

Table 2. Means of Embryogenic Cream Calli (EC) percentages as observed from Day 7 through to Day 35 in the 10 selected sorghum varieties

Genotype	**Name of**	**EC% (means of four treatments)**				
		Day	Day 14	Day 21	Day 28	Day 35
V 01	Alföldi 1	25.6	35.6	40.0	44.4	46.0
V 02	GK Emese	51.3	45.8	48.1	42.1	38.8
V 03	GK Zsófia	45.0	38.1	40.0	44.4	36.9
V 04	V4	38.8	35.0	35.6	38.8	34.4
V 05	V5	26.9	41.9	48.1	39.0	30.2
V 06	V6	37.3	42.3	46.7	43.1	36.5
V 07	V7	29.4	24.4	25.0	27.5	27.5
V 08	Farmsugro 180	51.9	52.9	51.9	45.0	43.1
V 09	Róna 1	39.8	35.2	30.8	26.7	29.4
V 10	GK Áron	27.5	45.6	45.6	45.6	31.3
P	0.697					

The effect of medium was the major source of influence on callus type and the change over time. As at day 35, the EC% means was higher than that of the other callus types. The EC% at day 35

for T1 were higher than and significantly different from those of treatment T2, T3 and T4. Treatment T3's EC% mean was significantly higher than that of T2 while that of T1 was the least at day 35. At day 7, T3 had a higher EC% mean than that of T1. The EC in T2 and T4 were less than those observed in T1 and T3, and they did not increase throughout the trial.

A major drawback to sorghum tissue culture remains the pigment exudation (phenolic compounds) from somatic cells (Liang et al. 1997) also referred by some authors as lethal browning (Malik and Saxena, 1992). The exudation is the reason that some calli in this study, even though embryogenic, turned brown, and therefore could not be candidates for regeneration. It is Liu et al. (2015) that reported lethal browning-free callus culture with a modified MS medium. Medium T1, which is a half strength MS medium containing 2 mgl^{-1} 2,4-D produced the highest percentage of EC calluses by day 28 and 35 of incubation. This medium differed from the modified MS medium by Liu et al. (2015) which they referred to as M11AP in that the latter was MS medium containing 1 mgl^{-1} 2,4-D, 1 gl^{-1} KH2PO4, 1 gl^{-1} L-asparagine, 1 gl^{-1} L-proline at pH 5.7. The M11AP medium was reported to have produced 84% EC albeit on a single genotype, SA281, higher than the amount produced by medium T1 in this study. Medium T3 produced the second highest EC% mean on both days 28 and 35, despite its mean being higher than that of T1 on both day 7 and day 14. This finding is not surprising considering that medium T3 contained 5 mgl^{-1} 2,4-D. The trend changes on day 21, where a high concentration of 2,4 -D appeared to have inhibitory effect on callus growth. At the same time, compared to cytokinin TDZ, growth hormone auxin 2,4-D was seen to have greater effect on callus induction.

3.3 Optimizing tissue culture media for sorghum somatic embryogenesis

The six media showed significantly different ($P<0.05$) average yellow callus percentages. Medium type E had a significantly higher average yellow callus percentage at 59.7% than medium D, A, B and C (in decreasing order) with average percentages 31.9, 27.6, 22.6 and 19.3, respectively. The control medium type F had a lower average yellow percentage than medium type E, but these two were not significantly different.

All the MS-based medium types had higher friable callus percentages which were significantly different from the non-MS-based types B, C and D. The half-strength MS medium type E and A had the highest average friable callus percentages at 25.1% and 22.1% respectively, which were not significantly different from that of the control medium F which had an average of 19.0%. The

non-MS-based medium types had very low average friable callus percentages at 5.6 % for medium B, 1.0% for medium C and 0.7% for medium D.

This study observed that half strength MS media supplemented with amino acids L-proline and L-asparagine, KH_2PO_4 and $CuSO_4 \cdot 5H_2O$ gave more yellow, and friable sorghum calli compared to full-strength media supplemented with the same components. This result gives more credence to the earlier findings that MS based media resulted in high callus induction rates (Liu et al. 2015), but also highlight the strength of MS media's effect on quality of the sorghum calli. The observation agrees with the findings of Wani et al. (2014), who reported sensitivity of explants on the strength of MS media, although their work was on *Costus pictus.*

The addition of components amino acids L-proline and L-asparagine, KH_2PO_4 and $CuSO_4 \cdot 5H_2O$ contributed significantly in maintaining yellow colour of the calli, a finding that corroborates the observation by Liu et al. (2015) who reported that addition of the components to MS media contributed to reduction of phenolics in sorghum tissue culture and led to increased callus induction rates. This observation is notable by the difference of medium type A which was MS medium without the components and medium types E and F, both of which had the additional components. The MS based media regardless of the strength were seen to produce friable calli, unlike the non-MS based media, although half strength medium types E and A resulted in slightly more friable calli than full strength medium type F. This result contrasts the findings of Elkonin et al. (1995) that friable (embryogenic) callus resulted on the addition of amino acids L-proline and L-asparagine, since there was no significant difference on average friable callus percentage between medium type A and medium types E, F considering that the former did not have the amino acids. Further, our findings diverge from the observation that Chu N6 medium resulted in more embryogenic calli than MS based media (Elkonin et al. 1995), although the Chu N6 medium used in this study did not contain the amino acids.

This study found that average yellow callus percentage was influenced by genotype whereby there were significant differences observed among both the varieties and culture media and therefore partly agree with the findings by Liu et al. (2015) that sorghum embryogenesis is genotype dependent. This result is however true only as far as the callus colour is concerned and not the average friable calli percentage, where there was no difference as a result of the varieties.

3.4 Doubled haploid production using an improved anther culture protocol for sorghum

Only four embryogenic calli were obtained from genotype 'GK Zsófia' on induction of the 36,000 anthers from the summer of 2018. The calli formed from anthers cultured on W14mf medium without 0.3 M mannitol pre-treatment, after 18 days of incubation in darkness, the initial 3 days being at 32˚C, while the rest were at 28±1˚C. The rest of the anthers discoloured and died on incubation for 30-35 days. All the four embryogenic calli recovered, regenerated on 190-2Cu medium under light condition at 25±1˚C for 20-30 days and formed green plantlets. The anthers from the winter of year 1 produced five embryogenic calli from genotype 'Róna 1' on W14mf medium without 0.3 M mannitol pre-treatment after 18 days of incubation in darkness, the initial 3 days being at 32˚C, while the rest were at 28±1˚C. Only one of the 5 embryogenic calli from the induction work of the winter of the first year regenerated and proliferated on 190-2Cu medium under light condition at 25±1˚C for 20-30 days, producing 5 shoots that were subsequently cloned to produce 5 individual green plantlets. Like the regenerants from genotype 'GK Zsófia' earlier obtained, the regenerants from 'Róna 1' produced shoots first, then followed by roots on the regeneration medium.

In the callus induction work of the summer of 2019, a total of 57 calli from 9 genotypes – 26 on induction medium M2, 17 on induction medium M3 and 14 on induction medium M1 were obtained. Out of these, 3 calli from 3 different genotypes ('ARET×VSZ25KKD'; 'AREL× SZE697/01' and 'AREL×ZSV04/30') regenerated on 190-2Cu medium under light condition at 25±1˚C for 20-30 days, with the regenerant from variety 'AREL×ZSV04/30' being an albino. Among all the regenerants, shoots formed before the roots on the regeneration medium. All the three regenerants proliferated and produced more than five shoots each, which were cloned and transferred to fresh 190-2Cu regeneration medium on which they were developed as individual plantlets.

Noteworthy, there was a significant increase in callus yield in the summer of 2019, where the induction media were M1, M2 and M3, and the anthers had been obtained from field grown plants, with a pre-treatment of 10˚C for 15 days in a lighted phytotron chamber at 85% relative humidity.

The ploidy assessment using SSR markers for 'Róna 1' resulted in monomorphic alleles for all the loci tested in the regenerants and polymorphic alleles for the diploid control (Tab. 3). The tests for

the ploidy level of the mentioned genotypes and all other subsequent regenerants in this study was confirmed using the direct method of flow cytometry.

Table. 3 Dimorphic and monomorphic allele sizes as obtained from polyacryilamide gel electrophoresis (PAGE) analysis for registered hybrid 'Róna 1' with SSR markers for the diploid control and regenerants.

Sample	**mSbCIR238**		**Xgap206**	
Genotype 'Róna 1 diploid control	108	126	75	83
Genotype 'Róna 1' Regenerant 1	126	126	83	83
Genotype 'Róna 1' Regenerant 2	126	126	83	83
Genotype 'Róna 1' Regenerant 3	126	126	83	83

Only one of the two 'Róna 1' regenerants that were treated with colchicine for chromosome duplication survived to heading, producing two additional tillers. The three non-colchicine treated regenerants produced at least 3 tillers each, subsequently producing panicles with varying quantities of seeds. The varying number of filled grains per panicle provided an opportunity to determine the fertility level of the individual plants: the non-colchicine treated plants grouped into 3 based on the visual observation of the seed quantity per panicle had 994, 146 and 24 as average number of grains per panicle, representing 80.5, 20.9 and 2.4% respectively as percentages of the total seed numbers per panicle (Fig. 3). The colchicine treated plants had an average of 144 grains per panicle, representing 9.03% of the total number of seeds per panicle.

Figure 3. Differential fertility levels recorded for genotype 'Róna 1' spontaneous di-haploid clones depicted by the number of seeds in a panicle, a., panicle with seeds in all the florets, b.,

panicle with seeds occupying partially the total number of florets, c., panicle with very few or no seeds in the florets: **ff** = fertile floret; **sf** = sterile floret.

Inherent tillering ability of sorghum (Lafarge et al. 2002), coupled with its high abiotic stress tolerance (Dalal et al. 2012) as was observed in this study, together with the appropriate induction and regeneration media, were found to be the greatest contributors to successful haploids production, if the culture conditions were set at optimum. The results of this study are a significant improvement compared to the works of Rose et al. (1986), Wen et al. (1991) and Kumaravadivel and Sree Rangasamy (1994) all of whom reported the production of haploids from one genotype each, albeit with some degree of uncertainty in the case of Wen et al. (1991). The success may be attributed to the W14mf basal medium that was used in the current study, when compared to the N_6 and MS-t-z-2 media that had been used in the studies mentioned, but which did not yield any callus when tested in the current study. Further, this study found that modification of medium W14mf with L-proline, L-asparagine and KH_2PO_4 led to maintenance of yellowish colour of the calli and contributed to reduced production of phenolics and therefore agreed with the findings of Liu et al. (2015); Sudhakar et al. (2009); and Chege et al. (2020).

Neither temperature pre-treatment nor osmotic starvation had any effect in callus induction in this study, rather, the collection of panicles when microspores were at mid to late uninucleate stage as in the work of Kumaravadivel and Sree Rangasamy (1994), just before the lateral expansion of the panicle caused the leaf sheath to split open was found to be more effective. Genotype dependency has been variously cited to impede sorghum callus induction (Sato et al. 2004; Raghuwanshi and Birch, 2010). Although embryogenic calli were induced for 9 genotypes in this study, genotype dependency was not overcome. Repeats of the induction with all the test materials always resulted in calli formation in all or some of these 9 responsive genotypes. Notably, the induction rates of the anthers from the summer season that were obtained from field grown crops were higher than those in the winter season obtained from crops grown in a glasshouse, therefore corroborating the study of Can et al. (1998), who reported 6.4% and 3.7% sorghum callus induction for field anthers and glasshouse anthers respectively.

This study established that when the calli were transferred on to the regeneration medium not more than 7 days after induction, there was more than 50% probability of regeneration. The ideal temperature for sorghum regeneration under light condition was 25˚C, which corroborated the findings of Kumaravadivel and Sree Rangasamy (1994).

4. CONCLUSION

The main focus of this project was on the development of an optimized protocol for production of sorghum doubled haploids. The technology has been relied upon to reduce the time of release of improved crop varieties through speedy fixation of alleles thus attaining homozygosity in improved germplasms in two seasons rather than the 8-10 years required in classical breeding. The speedy release of improved varieties can be considered a game-changer for sorghum breeding in a situation where new varieties have had a tendency of very slow delivery to the end users.

This project produced 4 confirmed doubled-haploid lines on W14mf medium or its modification with 1.0 g/l L-proline, 1.0 g/l L-asparagine and 1.0 g/l KH_2PO_4, from a total of 28 sorghum germplasms involved in the study. It was established that temperature and starvation pre-treatments did not provide any advantage to callis induction. Rather, collection of donor tillers at the right microspore development stage (mid to late uninucleate stage) was found to be more effective for callus inudction compared to the synchronization of micropore maturation effect of pre-treatemnt to callus induction. With regards to plant regeneration and rooting, the project successifully used 190-2Cu, and recommends that for success to be realized, calli should be transferred on to the regeneration medium less than 7 days after induction, and temperature maintained at 25˚C under light condition. The project reports that genotype dependency which has been variously reported to impede sorghum callus induction was not wholly overcome. The project found sorghum's high tillering ability and high abiotic stress tolerance to greatly contribute to attainment of haploid plantlets. Sorghum haploids were observed to spontaneously diploidize and produce seeds at rates upto 80.5%.

For somatic embryogenesis, it was established that half strength MS medium with 2 ml/l (0.1 mg/ml) 2,4-D with gelrite was the most effective resulting in more than 90% callus induction of sterile 1.5 day old germinating embryos. The addition of components amino acids L-proline and L-asparagine, KH_2PO_4 and $CuSO_4 \cdot 5H_2O$ contributed significantly to maintaining creamy colour of the calli. Phenolic exudation was greatly reduced if an explant was not from already differentiated tissues such as stems and leaves. An induction medium containing half strength MS and auxin 2,4-D (2 mg/l) as the plant growth regulator (PGR) produced a quality callus- friable and free from browning/ with reduced phenolic exudation.

5. NEW SCIENTIFIC RESULTS

1. Cluster analysis with SSR markers resolved 31 sorghum genotypes (15 Hungarian and 16 East African) into two major clusters at 16% similarity threshold; thus, a high level of genetic diversity among the genotypes from the two gene pools was observed.

2. Half strength MS medium modified with 1.0 g/L L-proline, 1.0 g/L L-asparagine and 1.0 g/L KH_2PO_4 contributed to quality callus (creamy in colour and friable) production when compared with full strength MS medium modified with the same compounds. The experiment established that half strength MS medium suppressed phenolic production in sorghum tissue culture experiments.

3. The addition of components such as amino acids L-proline and L-asparagine, KH_2PO_4 and $CuSO_4 \cdot 5H_2O$ contributed significantly to maintaining embryogenic character of the callus in the induction of somatic embryogenesis in sorghum tissue culture of diploid origin.

4. Lethal browning was reduced when the entire explant comprised of intercalating tissues on half strength MS medium supplemented with 2 mg/L 2,4-D in somatic tissue culture experiments.

5. The *in vitro* anther culture experiments successfully produced haploid plants from anther culture of five Hungarian germplasms on W14mf medium or its modification with 1.0 g/L L-proline, 1.0 g/L L-asparagine and 1.0 g/L KH_2PO_4, where pre-treatment was found not to provide any advantage to callus induction. Subsequently, four confirmed doubled-haploid lines were obtained. SSR analysis at dimorphic loci was applied to confirm haploidy and dihaploidy. The DH lines were passed to the breeding program.

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7. RELATED PUBLICATIONS

Peer-reviewed scientific articles

Chege, P., Kiss, E., Lantos, C., Palágyi, A., Pauk, J. (2021). Doubled Haploid Production Using an Improved Anther Culture Protocol for Sorghum [*Sorghum bicolor* (L.) Moench]. Phyton-International Journal of Experimental Botany, 90(2), 475–487. doi:10.32604/phyton.2021.013557

Chege, P., Lantos, C., & Pauk, J. (2020). Retrospect on *in vitro* androgenesis of sorghum (*Sorghum bicolor*). Plant Breed, 00, 1– 9. https://doi.org/10.1111/pbr.12843

Chege, P., Palágyi, A., Lantos, C., Kiss, E., & Pauk, J. (2020). Improved Culture Media for Embryogenic Callus Generation in Sorghum [*Sorghum bicolor* (L.) Moench]. Phyton-International Journal of Experimental Botany, 89(1), 111-119. doi:10.32604/phyton.2020.07554

Conferences

Abstract and talk at: XXIVth EUCARPIA Maize and Sorghum Conference, Munich, Germany, October 7-9, 2019; Topic- Diversity analysis of some selected Hungarian and East African sorghum [*Sorghum bicolor* (L.) Moench] genotypes using SSR markers

Abstract, poster and talk at 2nd European Sorghum Congress, Milan, Italy, November 7-8, 2018; Assessing various tissue culture aspects (Mainly 2,4-D and TDZ) for efficient somatic sorghum [*Sorghum bicolor* (L.) Moench] embryogenesis

Other scientific articles with other topics published during the PhD program

Kanbar, O., **Chege, P.,** Lantos, C., Kiss, E., & Pauk, J. (2020). Characterization of winter wheat (*Triticum aestivum* L.) germplasm for drought tolerance. Plant Genetic Resources: Characterization and Utilization, 1-13. doi:10.1017/S1479262120000398

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Gyulai, G., **Chege, P.,** Guylai, G.Z., & Foshee, W.G. (2019) Genetic analysis of common millet (*Panicum miliaceum*) heirlooms to select climate adaptive millet cultivars. In: Comtois, N.F. (ed): Millets: Properties, Production and Applications. Nova Science Publisher, Inc. New York, USA. Chapter 3. pp. 47-55. ISBN: 978-1-53614-692-9