Hungarian University of Agriculture and Life Sciences

Doctoral School of Plant Science

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

Ph.D. Dissertation

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Abbreviations

°C – Degree Celsius, centigrade
µl- Microliter
2,4-D- 2,4-Dichlorophenoxyacetic acid
ANOVA- Analysis of Variance
B.C. – Before Christ
BAP - 6-Benzylaminopurine
bp- Base pairs
CIRAD- French Agricultural Research Centre for International Development
cm – Centimetres
CV- Coefficient of Variation
DH – Doubled Haploids
DMSO- Dimethyl sulfoxide
DNA- Deoxyribonucleic acid
dNTPs- Deoxyribonucleoside Triphosphate
dsDNA- double stranded DNA
E- Experimental hybrid
EB -Embryogenic Brown
EC- Embryogenic Cream
ELS- Embryo Like structure
F_1 – First filial generation
F_2 – Second filial generation
F_5 – Fifth filial generation
FAO - Food and Agriculture Organization
FeEDTA- Ferric Ethylene Diamine Tetraacetic acid
Ff- fertile floret
G - Grams
G_1- The phase immediately after mitosis in cell cycle
G_2- Growth and preparation for mitosis phase of cell cycle
GC- Germ Cell
GK- Gabonakutató (Cereal Research Non-profit Ltd)
He- Expected heterozygosity
Ho- Observed heterozygosity
IAA – Indole Acetic Acid
ICRISAT- International Crops Research Institute for the Semi-Arid Tropics
INTSORMIL- International Sorghum and Millet Collaborative Research Support Program
Kb - Kilo base pairs
kg/ha – Kilograms per hectare
m- Meter
M- 1 Molar concentration
M- Mitosis phase of cell cycle
MAS – Marker Assisted Selection
Mb – Mega base pairs
MGU – Male Germ Unit
mm- Millimeter

MS – Murashige and Skoog
NAA- Naphthalene Acetic Acid
NARS- National Agricultural Research Systems
NEB -Non-Embryogenic Brown
NES- Non-Embryogenic Soft
ng/µl- Nanograms per microliter
PAGE - Polyacrylamide Gel Electrophoresis
PCR- Polymerase Chain Reaction
PGR – Plant Growth Regulator
pH- Acidity measurement scale
PIC- Polymorphic Information Content
PMI- Pollen mitosis I
PMII- Pollen Mitosis II
PVC- Polyvinyl Chloride
R- Regeneration media
R- Released hybrid
RAPD – Random Amplified Polymorphic DNA
RCBD- Randomized Complete Block Design
RFLP- Restriction Fragment Length Polymorphism
S – DNA replication phase of cell cycle
SAT – Semi-Arid Tropics
SC- Sperm Cell
Sf- Sterile floret
SNP – Single-Nucleotide Polymorphism
SSR - Simple Sequence Repeat
T- Treatment
T/ha – Tonnes per hectare
TBE- Tris-Borate-EDTA
TDZ- Thidiazuron
TEMED - Tetramethylethylenediamine
UPGMA- Unweighted Pair Group Method with Arithmetic mean
UPOV - International Union for the Protection of New Varieties of Plants
USDA- United States Department of Agriculture
UTR- Untranslated region of DNA
UV- Ultraviolet
V- Volts
VC – Vegetative Cell
W/V- Weight (of solute) per volume (of solvent)

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.

The experiments undertaken in this work, in addition to establishing the genetic relatedness of Hungarian germplasm with some selected East African germplasms were undertaken with the hope of contributing to the improvement of several aspects of sorghum somatic tissue culture, that would in turn contribute to the possibility of successfully developing doubled haploid plants to maturity. These plants would be expected to achieve homozygosity in a short period as opposed the classical breeding's eight or more generations. At the same time, critical information on attainment of spontaneous haploids would be obtained.

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. OVERVIEW OF LITERATURE

2.1 Sorghum domestication

Sorghum [*Sorghum bicolor* (L.) Moench] is a cultivated diploid (2n=20), tropical cereal, C4 grass with a high photosynthetic efficiency and inherent high biomass yield. It is difficult to determine when and where domestication occurred (de Wet et al. 1970). Murdock (1959) has suggested that the Mande people around the headwaters of the Niger River may have domesticated sorghum. Doggett (1970) indicated that archaeological evidence suggests that the practice of cereal domestication was introduced from Ethiopia to Egypt about 3000 B.C. It is possible that domestication of sorghum began about that time. De Wet et al. (1970) studied archaeological reports but found only meager information about sorghum. De Wet and his colleagues suggest that sorghum had a diverse origin and probably arose from *Sorghum verticilliflorum*. *S. arundinaceum* is a grass of the tropical forests, and *S. aethiopicum* and *S. virgatum* are found in desert regions. These habitats are outside the major sorghum areas and probably contributed less to its domestication. *S. verticilliflorum* is usually found in areas where sorghum is cultivated.

There is tremendous variation in *S. verticilliflorum*: and it, as well as the other wild species, readily crosses with cultivated sorghum. It yields well and was probably collected and used before the advent of agriculture. Snowden (1936) and Porteres (1951) suggested that races *durra*, *guinea*, and *caffra* are closely allied and may have arisen from *S. aethiopicum*, S. *arundinaceum*, and *S. verticilliflorum*, respectively. This, however, is difficult to demonstrate experimentally. Morphological differences between races may have arisen because of ethnic isolation. *Caffra* is widely grown in Bantu Africa, while *durra* is not found there. *Caudatums* are most common in central Sudan, and Guinea corns are found primarily in West Africa. Distribution indicates that the races *caffra* and *caudatum* derived from *S. verticilliflorum*, and that *durra* possibly could have come from *S. bicolor*. *Guinea* corn is quite distinct, but it is questionable that it could have come from *S. arundinaceum* (which is a grass of tropical forests and not found where sorghum is extensively cultivated).

Introgression studies indicate that cultivated sorghums probably developed through disruptive selection (Doggett 1965). Crossing easily occurs between wild and cultivated types; however, these types form distinct populations. It is speculated that, as man began to select, there was substantial gene flow between improved and unimproved types. This gene flow would decrease as field sizes became larger. The selection by man and nature would provide a disruptive force resulting in diverse populations (polymorphic populations). These disruptive forces have been continuously active through time (and are still active), influencing cultivated and wild populations.

Most intermediate forms do not exist long in nature: those backcrossed by cultivated crops would tend to contribute genes in the direction of cultivated types; and those backcrossed by wild types would tend to contribute genes to the wild population. Polymorphic populations would tend to be maintained and change over the years. New forms would arise, leading to the types of sorghum now in cultivation. Ethnic isolation would help this process.

The process of domestication involved a change in several characteristics of the plant. A tough primary axis (rachis) and persistence of the sessile spikelet were probably introduced early in the process. It is likely that the transformation of a loose and open inflorescence into a compact type involved several changes: first, an increase in the number of branches per node; second, an increase in the number of branches per primary inflorescence branch; and third, a decrease in the internode length on the rachis . An increase in seed size also was probably a product of domestication, the seed becoming large enough to protrude from the glumes (House 1985).

2.2 Sorghum taxonomy

2.2.1 Classification

Sorghum belongs to the family Gramineae, sub family *panicoideae* and the tribe *andropogonea* (Dillon *et al.* 2007). It is believed that there are 20 to 30 species of the genus *Sorghum* that are recognised. The wild sorghum includes: *halepense* (L) pers, *S. propinquum* (kunth), *S. bicolor* sub-species *drummondii* and *S. bicolor* subspecies *Verticilliflorum*. Cultivated germplasm has been classified into five major races *Bicolor, Caudatum*, *Durra*, *Guinea* and *Kafir*, with ten intermediate races based on panicle morphology. Harlan and de Wet (1972) classified, the *Bicolor* race based on loose panicle with grains covered by large, closed glumes, commonly distributed in Asia and Africa. According to Harlan and de Wet (1972), the *Caudatum* race is characterised by symmetrical grain, flattened on the ventral surface and convex on the dorsal surface.

The panicle morphology structure varies with shape (Fig.1). This race is mainly found in Central and East Africa. The *Durra* sorghums have very compact panicles with curved pendulous and tiny glumes that are attached to globular grain. This race is common in East Africa, the Middle East and India. The *Guinea* sorghums are tall with loose panicles, spikelet with open glumes enclosing an elliptical grain and are photo period sensitive. These cultivars are found in West Africa. The *Kafir* varieties are the smallest sorghums with relatively compact and cylindrical panicles consisting of symmetrical grain flattened on the ventral surface and convex on the dorsal. This race is grown mostly in Eastern and South Africa. Morphological intermediate races such as

Durra-caudatum, *Bicolor-caudatum*, *Guinea-caudatum* have been reported in Ethiopia intermediates (Mekbib, 2009).

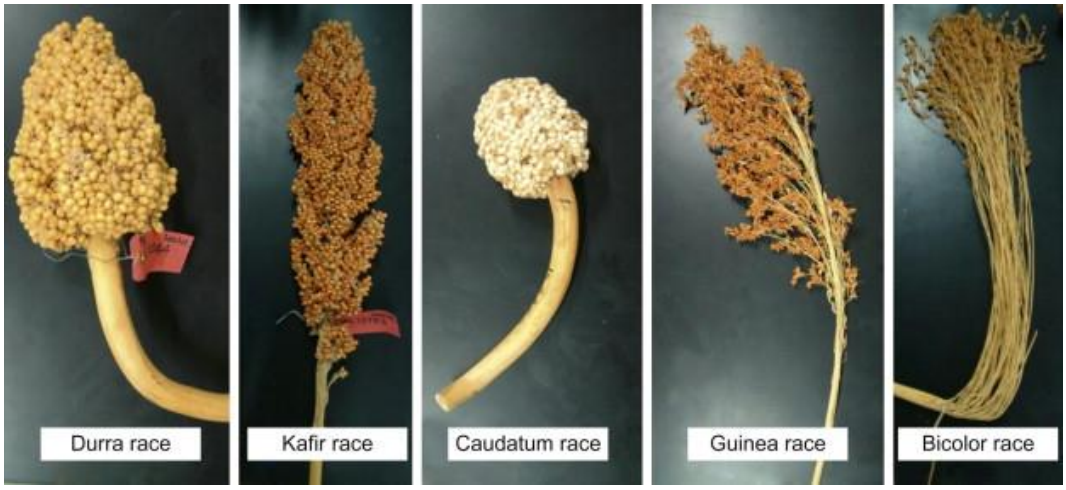

Figure 1. The sorghum basic races by their spikelet morphology (Harlan and de Wet, 1972)

2.2.2 Utilization-based classification of sorghum

In several regions of the world and most notably in Europe, sorghum is variously classified based on its utilization as: grain sorghum, forage sorghum, Sudan grass, sorghum-Sudan grass and sorghum-almum. Grain sorghum comprises of dwarf varieties, growing upto 2 to 5 ft, suitable for grain harvesting/ combining. Forage sorghum grows 6 ft to 12 ft tall, producing a much higher mass of dry matter than grain sorghum, and is suitable for silage use. Sudangrass on the other hand are fine stemmed, short season varieties grown to supply pasture or green feed during mid-summer when perennial grasses are dormant. Sorghum-sudangrass hybrids are a cross between the two forage types, that have intermediate yield potential and can be used for pasture, hay or silage. Sorghum almum is a hybrid between grain sorghum (*S. bicolor*) and Johnsongrass (*S. halepense*). It is stout amd can grow upto 14 ft tall. Its stems grow from extensively creeping rhizomes and is utilized for pasture or breeding for forage and grain sorghum (Undersander et al. 1990).

2.2.3 Sorghum genome

Sorghum is a diploid (2n=20) with a genome size of 710 Mb, with approximately 33,000 genes (Sagnard et al. 2011). The small genome makes the crop an attractive model for functional genomics of saccharinae and other C4 grasses (Wang and Peterson 2012). According to Paterson et al. (2009), genetic variation in the partitioning of carbon into sugar stores versus cell wall mass, and in perenniality and associated features such as tillering and stalk reserve retention, make

sorghum an attractive system for the study of traits important in perennial cellulosic biomass crops. Its high level of inbreeding makes it an attractive association genetics system.

The number and sizes of sorghum gene families are similar to those of *Arabidopsis*, rice and poplar (Paterson et al. 2009). According to Paterson et al. (2009), a total of 9,503 (58%) sorghum gene families are shared among all four species and 15,225 (93%) with at least one other species. Nearly 94% (25,875) of high-confidence sorghum genes have orthologues in rice, *Arabidopsis* and/or poplar, and together these gene complements define 11,502 ancestral angiosperm gene families represented in at least one contemporary grass and rosid genome (Paterson et al. 2009). Paterson et al. (2009) explains that 3,983 (24%) gene families have members only in the grasses, sorghum and rice while 1,153 (7%) appear to be unique to sorghum.

2.3 Morphology of sorghum

2.3.1 Roots

The root system of sorghum is extensive, and there are many root hairs (almost twice that of maize, for example). An embryonic or primary root first appears upon germination. Several such roots develop; these are not branched or are sparsely branched. Secondary roots develop from the first node; it is these roots that develop into the extensive root system of the plant. The primary roots subsequently die. Brace roots may appear later on the lowermost nodes and may be numerous if the plant is un-adapted. These roots are not effective in uptake of water and nutrients. The cultivated sorghums are either non-rhizomatous or very weakly rhizomatous and are annual or (weakly) perennial. The root system, however, survives to support the development of ratoon crops (a second, third, or more growth of culms from the same root system) from adventitious buds at the base of the parent stem. Well-developed rhizomes are found only in the subspecies *halepense* (Johnson grass) (House 1985).

2.3.2 Culms

The culm, or stem, is made up of a series of alternating nodes and internodes. The stem is slender to very stout, measuring 0.5 to 5 cm in diameter near the base, becoming narrower at the upper end, and varying in length from 0.5 to 4 m. It is solid, with a hard cortex or rind and a softer pith. Vascular bundles are scattered throughout the stem, but there are more near the peripheral area, where they are so closely associated that they form almost a solid ring. The vascular bundles in the central portion of the stem are larger than those at the periphery. The central bundles branch into leaf midribs, while the peripheral bundles branch to form the smaller veins in the leaf blade. The pith may be sweet or insipid, juicy or dry. In older stems the pith may crack, especially if dry.

The node appears as a ring at the base of the leaf sheath; this is the point at which the leaf is attached to the stem (also the point at which brace roots develop). There is a complex anastomosis of vascular bundles from the stem to the leaf at this point. A bud forms at each node, except at the node to which the flag leaf is attached. These buds, at successive nodes, arise on alternating sides of the stem. At times, these buds will develop to form axillary tillers. Basal tillers, if any, form at the first node (House 1985).

2.3.3 Leaves

Leaves are variously distributed along the stem in sorghum; in some types they may be concentrated near the base, while in others they are uniformly distributed. Leaves develop at different angles to the stem, varying from almost vertical to near horizontal. The leaf blade may be straight, or may slowly loop over, forming an arc. The tip of the leaf may even drop down. The leaves vary in length, usually being shorter and smaller at the top (the top leaf is called the flag leaf); in the lower mid-section, they may be as long or slightly longer than those at the base of the plant. Leaves may be 1 m long and may vary in width from 10 to 15 cm. The plants vary greatly in number of leaves: in well-adapted plants there are usually 14 to 17 leaves, but less adapted plants may have as many as 30 leaves. Generally, the embryo in the seed will have five to seven embryonic leaves, the greater number being found in the more mature seeds. A sorghum seed reaches physiological maturity in about 30 days and at this time has six or seven embryonic leaves. Approximately the same amount of time (4 to 5 days) is required to lay down a leaf in the embryo as in the vegetative growing point, the meristem.

The leaves develop alternately in two ranks along the stem and consist primarily of a sheath and a blade. The sheath is attached to a node and surrounds the internode, and frequently the node above it, before the blade extends outwards. Frequently, the sheaths attached to lower nodes will cover the nodes above, but those higher on the plant will not extend as far as the node above. This sheath is frequently covered with a waxy bloom; at times the bloom is quite pronounced. The blades are broad at the base and taper upward to a fine point; they are glabrous, except on the inside just above the ligule and on the outside near the junction with the sheath. The margins of the leaf are smooth or scabrid, especially on the upper half. The midrib is prominent, greenish or white, flattened or slightly concave on the upper surface and convex on the lower one. The blades are thicker at the base than at the tip and along the midrib than along the margins. When damaged, the injured spot will turn tan, red, or deep purple (almost black), depending on the plant colour. There is a short (1 to 3 mm) membranous ligule at the junction of the leaf blade with the sheath. Leaves

of the wild species are frequently long (30 to 75 cm) and slender (0.5 to 7 cm in width) (House 1985).

2.3.4 Inflorescence

2.3.4.1 Panicle

The panicle may be short and compact or loose and open: 4 to 25 cm or longer, and 2 to 20 cm or wider. The central axis of the panicle, the rachis, may be completely hidden by the density of the panicle branches or completely exposed. The rachis differs greatly in its shape and length—from long and thin to short and stubby. The rachis may be striated (frequently channelled), and it may be hairy or glabrous. Several branches develop at each node, and these branches vary in length, may be stout or slender, rigid, or flexible, hairy or almost glabrous, branched beginning near the base or not branched until near the tip. The panicle usually grows erect at the apex of the culm but may be recurved. The wild and weedy sorghums have a rather loose panicle with spreading branches. The panicle is often large and pyramidal in shape (House 1985).

2.3.4.2 Raceme

The raceme always consists of one or several spikelets. One spikelet is always sessile and the other pedicellate, except the terminal sessile spikelet, which is accompanied by two pedicelled spikelets. The racemes vary in length according to the number of nodes and the length of the internodes. There are 1 to 4 nodes in some species, and 5 to 8 nodes in others; internodes vary in length, thickness, and hairiness, depending on the species. On the pedicelled spikelets, the pedicels vary in length from 0.5 to 3.0 mm, and usually are very similar to the internodes.

Sessile spikelets: The sessile spikelet varies in shape from lanceolate to almost rotund and ovate and is sometimes depressed in the middle. The colour is green at flowering, changing to shades of straw, cream, buff, yellow, red, brown, purple, or almost black at grain maturity. The intensity and extent of colouring on the glumes is variable. Glumes vary from quite hairy to almost hairless. Some species have thin and brittle glumes, while others have thin and papery ones. The lower glume is usually somewhat flattened and conforms to the shape of the spikelet, while the upper one is more convex or boat shaped. The seed may be enclosed by the glume or may protrude from it, being just visible to almost completely exposed.

There are two lemmas, each a delicate white tissue easily overlooked on a casual glance. The lower lemma is elliptic or oblong, about equal in length to the glume; the upper lemma is shorter, more ovate, and may be awned. There are also two lodicules and a palea, but these are much reduced

and are of little interest. Sorghum has two pistils and three stamens. Each fluffy stigma is attached to a short stout style extending to the ovary. The anthers are attached to long threadlike filaments (House 1985).

Pedicelled spikelets: These are much narrower than the sessile spikelets, usually lanceolate in shape. They may be smaller, the same size, or longer than the sessile spikelets. They are male or neuter in sex, but (very rarely) may have a rudimentary ovary. The lemmas are much reduced in size and only rarely does the upper lemma have an awn.

2.3.4.3 Seed or caryopsis

Seeds are spherical in shape, varying to somewhat flattened on one side (turtle-backed). They range tremendously in pericarp colour (red, brown, white, yellow, cream) and have either a dull or pearly lustre. The testa may also be coloured, usually a dark red to dark brown. The endosperm is usually white, though it may be yellow. Yellow endosperm colour is due to carotenoid pigments that have a relatively low vitamin A activity. There often are two distinct lines extending from the apex to the base of the seed. The embryo mark (scutellum) varies in length from about one-half to two-thirds the length of the grain, and is elliptic to elliptic oblong, concave to flat, or (rarely) convex. The hilum is at the base on the side opposite the embryo. The hilum frequently turns dark at about the time the seed reaches physiological maturity. The endosperm varies from soft with little corneous portion to a solid corneous seed condition. Seed size varies from very small (less than 1 g/100 seeds to large (5 to 6 g/100 seeds) (House 1985).

Morphological descriptors are used to distinguish one genotype from another based on their traits and helps in understanding the physical similarities and differences among the genotypes (Ngugi and Maswili, 2011). Phenotypic characterisation is usually based on both qualitative and quantitative morphological characters (Upadhyaya et al. 2010). The qualitative traits in sorghum include leaf midrib colour, grain colour, glume colour, endosperm colour texture, pericarp colour, leaf trichomes, testa colour, pericarp thickness and panicle compactness (Mofokeng et al. 2017). Quantitative traits are important in determination of genetic diversity among genotypes (Latif 2011). Quantitative traits in sorghum include plant height, time to maturity, leaf area, leaf width, leaf length, number of leaves, panicle length, grain yield per plant, grain size, 1 000 grain seed weight, grain number per panicle, panicle width, number of primary branches per panicle and panicle weight (Rami et al. 1998).

2.4 Growth stages and morphology of sorghum

2.4.1 Vegetative phase

2.4.1.1 Germination and seedling development

As explained by House (1985), when a sorghum seed is placed in moist soil, it takes up water and swells with germination occuring quickly, in warm soils (20 °C or above) the coleoptile first appearing above the ground after 3 or 4 days and upto 10 days, in colder soils (13 to 20 °C). As the seed swells the seed coat breaks, and a small coleoptile and the primary root (radicle) emerges (House 1985). The coleoptile grows longer and several more primary roots appear. The coleoptile begins to emerge from the ground, and the first leaf breaks through the tip. The young plant begins to grow, adding more leaves, and the coleoptile remains as a sheath at the base of the plant.

The mesocotyl grows during this period, and a node is formed at the base of the coleoptile just below the ground line. Secondary roots begin to develop from this node when the plant is 3 to 7 days from emergence. The young seedling is using nutrients stored in the endosperm during this period. About the time the secondary roots have begun to develop, the mesocotyl begins to die and the major root system develops from the secondary or adventitious roots. Some sorghums tiller profusely, especially the Sudan grasses and forage sorghums. The grain sorghums vary in their capacity to tiller, but usually do so only if there is adequate moisture or a poor stand. In normally tillering varieties, tillers develop from adventitious buds at the basal node soon after the secondary roots develop. Heads on the main stem flower at about same time as those on the tillers, or the heads on the tillers may flower later. The plant remains in a vegetative phase for about 30 to 40 days, during which all leaves are formed. After this period, growth occurs by cell elongation (House 1985; Vanderlip and Reeves, 1972).

2.4.2 Reproductive phase

2.4.2.1 Inflorescence development and fertilization

The floral initial forms 30 to 40 days after germination (but floral initiation may range from 19 to 70 days or more). Usually, the floral initial is 15 to 30 cm above the ground when the plants are some 50 to 75 cm tall. Floral initiation marks the end of the vegetative growth due to meristematic activity. The grand period of growth in sorghum follows the formation of a floral bud and consists largely of cell enlargement. During the period of rapid cell elongation, the floral initial develops into an inflorescence. About 6 to 10 days before flowering, the boot will form as a bulge in the sheath of the flag leaf. This will occur, in a variety that flowers in 60 to 65 days, about 55 days

after germination. Sorghum usually flowers in 55 to 70 days in warm climates, but flowering may range from 30 to more than 100 days. The sorghum head begins to flower at its tip and flowers successively downward over a 4- or 5-day period (Srinivasa-Rao et al. 2013a). Because all heads in a field do not flower at the same time, pollen is usually available for a period of 10 to 15 days.

At the time of flowering, the glumes open and the three anthers fall free, while the two stigmas protrude, each on a stiff style. Flowering frequently occurs just before or just after sunrise but may be delayed on cloudy damp mornings. The anthers dehisce when they are dry (but not in heavy dew or rain) and pollen blows into the air. Sorghum is primarily self-pollinated (about 2 to 10% or more cross-pollination); that is, the pollen from a head fertilizes most of the eggs on the same head. The pollen drifts to the stigma, where it germinates, and the pollen tube, with two nuclei, grows down the style. The two sperm cells fertilize the egg and the central cell, forming a 2n zygote and a 3n endosperm, respectively. Sorghum has a 20-chromosome complement. The glumes close shortly after pollination, though the empty anthers and stigmas still protrude (except in the long-glumed types). The florets of some of the very long-glumed types do not open for fertilization-a phenomenon known as cleistogamy.

Cytoplasmic male sterility has been found in sorghum and has made possible the development of a hybrid seed industry. A good male-sterile plant will not develop anthers, but in some instances dark-coloured shrivelled anthers with no viable pollen will appear. Partially fertile heads are also observed, and although the anthers frequently have viable pollen, the quantity is less than in normal plants. Viability of pollen in partially fertile plants is an important problem for seed producers (Srinivasa-Rao et al. 2013b)

2.4.3 Maturity phase

2.4.3.1 Seed development

The ovule begins to develop as a light green, almost cream-coloured sphere; after about 10 days it begins to take size and becomes a darker green. It takes about 30 days for the seeds to reach maximum dry weight (physiological maturity). During this development, the seed passes through three stages; (1) "milk, " (2) "early dough," and (3) "late dough." These terms, while commonly used, are not specifically defined. The seeds begin to turn from green to the colour that they will be at maturity. The seeds contain about 30% moisture at physiological maturity; they dry to about 10 to 15% moisture during the following 20 to 25 days. During this period, they lose upto 10% of their weight. The seed is ready for harvest at any time from physiological maturity to seed dryness; however, seed with more than 12% moisture must be dried before storage. It is easy to recognize

the pericarp, the endosperm, and the embryo in a sliced mature dry seed. Lower leaves begin to die and dry up during this period. By the time the grain begins to dry, four or five of the lower leaves may dry up and drop from the plant. There is a distinct varietal difference in the rate of senescence of remaining leaves. All leaves may be dried, or almost dried, at grain maturity; or the plant may remain green (House 1985).

2.5 Some agronomic aspects of sorghum

2.5.1 Adaptability

Sorghum adapts to many environments, requiring 90 to 140 days to mature (Wani et al. 2011). Highest yields are usually obtained from varieties maturing in 100 to 120 days (Hermuth et al. 2016; House 1985. According to House (1985), such grain sorghum usually has a grain-to-straw ratio (harvest index) of about 1:1. House (1985) opines that varieties maturing earlier may not yield quite as much because of the reduced growing period; late-maturing varieties tend to put on foliage and make less grain (the grain-to-straw ratio may run as low as 1:5). Better yields of such late varieties commonly average 1500 to 2000 kg/ha, compared with 4000 to 5000 kg/ha or more for 100 to 120-day types (House 1985).

2.5.2 Fertilizer response

As explained by House (1985), fertilizer response for sorghum varies for different varieties. Many traditional varieties developed in low fertility and drought situations produce 6 to 10 kg grain per kg applied nitrogen, whereas varieties responsive to high levels of fertility produce 20 to 40 kg grain per kg applied nitrogen (House 1985; Ajeigbe et al. 2018).

2.5.3 Water relations

Sorghum is usually grown under hot, dry conditions (House 1985). According to Assefa et al. (2010), the plant roots penetrate a greater volume of soil to obtain moisture. House (1985) elucidates that sorghum requires less moisture for growth than some other cereal crops: 332 kg of water per kg of accumulated dry matter; maize requires 368 kg of water; barley, 434 kg; and wheat 514 kg. According to House (1985), sorghum tends to "hang on" during the dry period and resumes growth with the return of rain. Assefa et al. (2010) in their study demonstrated that water requirement of sorghum increases as the plant grows, reaching a peak during the flowering period after which the moisture consumption decreases. House (1985) pointed out that at peak consumption, sorghum uses about 6 to 7 mm of water per day. Sharma et al. (2019) in agreement with House (1985) pointed out that sorghum has also the capacity to withstand wet extremes.

2.5.4 Temperature relations

As explained by Tack et al. (2017) sorghum is able to give yield to grain even when temperatures are high. As House (1985) reports, at elevated temperatures, 40 °C and above, accompanied by low relative humidity of 30% or less, crossing is a bit difficult, but grain yield may be obtained if moisture is available, especially prior to and during the flowering period. Floral development and seed set are normal at temperatures of 40 to 43 °C and at 15 to 30% relative humidity, if soil moisture is available (House, 1985). On the other hand, Yu et al. (2004) in agreement with House (1985) points out that sorghum is not as tolerant as maize to cool weather. According to Patanè et al. (2006), the crop the germination and growth of the crop is dependent upon the attainment of an optimum soil temperature (15-20°C).

2.5.5 Plant protection

2.5.5.1 Insects

As Guo et al. (2011) indicates, insects are a serious problem in the cultivation of sorghum. The crop is highly vulnerable to shoot fly (*Atherigona soccata*) damage in the initial stages of its development as reported by Gorthy et al. (2017). Stem borers are also devastating pests for the crop (Berg and Rensburg, 1991). Sorghum midge (*Contarina sorghicola*) has been described to cause significant damage during seed set (Strachan et al. 1993). As House (1985) explains, midge lays its egg in the floret at the time of flowering and the maggot feeds on and destroys the developing seed, which can lead to complete crop loss.

2.5.5.2 Diseases

Several diseases have been reported to be of major economic importance in sorghum. Some of these include grain molds (Thakur et al. 2006); downy mildew (*Peronosclerospora sorghi*) (Bock and Jeger, 2002); and charcoal rot (*Macrophomina phaseolina*) (Odvody and Dunkle, 1979). Some diseases such as anthracnose (*Colletotrichum graminicola*) (Mathur et al. 2002), downy mildew (Jeger et al. 1998), and maize dwarf mosaic virus (Seifers and Karr, 2011) are also among those of epidemiological importance, especially in the temperate zones. House (1985) recommends breeding for resistance as the best method of control for sorghum diseases.

2.5.5.3 Weeds

The parasitic higher plant Striga (witchweed) is a major limiting factor in sorghum production (Runo and Kuria, 2018). Temesgen (2019) opines that *Striga hermonthica*, found across the major

sorghum growing regions of the African continent, is the most important species. House (1985) describes *Striga asiatica* as the most important species in India.

2.5.5.4 Bird damage

Birds have been variously described as a major menace to sorghum production, more importantly when the crop is introductory or a variety that matures much earlier or later than the local type (Duncan 1980; House 1985). House (1985) among other researchers advises that large sorghum acreage and ensuring synchronization in the maturation of crops in the field may significantly contribute to limiting bird damage.

2.5.5.5 Nematodes

According to Talwana et al. (2010), nematode problems have been found in cereal crop fields indiscriminately, their occurrence increasing with high crop intensity especially in areas with sandy soils.

2.6 Utilization of sorghum

2.6.1 Human food use

Sorghum is a staple cereal grain for many of the world's most food insecure people (Tari et al. 2012). According to Srinivasa-Rao et al. (2014) it is the fifth of the most consumed food crops in the world after rice, wheat, maize and potatoes. According to Hulse et al. (1980), sorghum is a good staple food crop due to its high nutritive value: It is rich in starch, proteins, micronutrients and crude fibre but low in fat. The crop has a protein level ranging from 7.3-15.6% (Hulse et al. 1980), which is higher than that of most other cereals consumed by humans (Ahmed et al. 1996). Dykes and Rooney (2006) report that sorghum is a good source of phenolic compounds, which provide several important health promoting properties: prevention and reduction of oxidative stress, anti-cancer, anti-diabetic, anti-inflammatory, anti-hypersensitivity and cardiovascular disease prevention. However, it has a high protein indigestibility (Weaver et al. 1998).

As Zabala et al. (2015) elucidates, in many parts of the world, sorghum has been used traditionally in food products and various food items: porridge, unleavened bread, cookies, cakes, couscous, and malted beverages are made from this versatile grain. These varied foods have differential preparation methods depending on the region (Zabala et al. 2015). Young et al. (1990) described boiling as one of the most basic preparation methods and tested various cultivars for ease of the process. Ratnavathi and Patil (2013) proposed that sorghum grain may be ground into flour or decorticated before grinding to produce either a fine particle product or flour, before preparation

into various traditional foods. Adebo (2020) has described the various ways through which grain sorghum has been fermented to produce various products such as beer, sorghum malt and meal. The crop has received considerable attention as its protein digests have been shown to be safe and tolerable to patients of celiac disease, which has high prevalence in Northern Europe (Ciacci et al. 2007).

2.6.2 Livestock feed use

Sorghum is also important an animal feed (Nasidi et al. 2019). A review by McCuistion et al. (2019) on comparisons of the dietary value of various grains demonstrated that some sorghums with a nutritional feeding value equivalent to that of maize are available. As explained by Safaei and Yang (2017), techniques such as grinding, crushing, steaming, steam flaking, popping and extruding may be used in processing sorghum to improve its feed value. The products are then fed to beef and dairy cattle, laying hens and poultry and pigs, and are used in pet foods (Ratnavathi and Komala, 2016). Compared to other grains, several studies have shown a reduced occurrence of mycotoxins such as aflatoxin in grain sorghum (Matumba et al. 2011). Where maize fails because of high temperatures and insufficient rainfall, sorghum serves as an important alternative source of fodder according to Getachew et al. (2016).

2.6.3 Industrial use

Sorghum has been shown to have a significant industrial importance in the manufacture of wallboard, biodegradable packaging materials and low-cost adhesives (Rooney and Waniska, 2000). Zhan et al. (2003) opined that sorghum has the potential for bio-industrial applications such as the production of ethanol and lactic acid. According to Diao et al. (2002), sorghum can be converted into activated carbon and as pointed out by da Silva and Taylor (2004), its proteins can be extracted for commercial bran production.

2.7 Current production trends

Sorghum is one of the most important cereal crops globally and it is ranked fifth in cultivation area after wheat, maize, rice and barley (Srinivasa-Rao et al. 2014). USDA presented the average global productivity in 2016/2017 as 1.46 t/ha (The Odum Institute 2017). However, productivity in some countries such as Argentina (4.86 t/ha), the USA (4.89 t/ha) and Egypt (5.36 t/ha) was well above the average. In the Semi-Arid Tropics (SAT) where it is a dietary staple for more than 500 million people, countries in the region including Burkina Faso, Mali, Mozambique, Nigeria, Sudan, and Tanzania to name but a few had meagre yields (t/ha) of 0.93, 1.12, 0.60, 1.26, 0.73 and 1.00, respectively, which were below the global average. In this reporting

period (The Odum Institute 2017), the crop's yields in other African countries, namely Ethiopia, Niger, Cameroon, Uganda, Ghana, South Africa were (t/ha) 2.00, 0.49, 1.53, 0.91, 1.04 and 3.22, respectively. The yield (t/ha) in India and Pakistan were recorded as 0.78 and 0.58 while that in China, their Asian counterpart was reported as 4.78, making it to the top 5 world major producing countries. The European Union countries among them Italy, France and Hungary recorded an average yield of 5.42 t/ha, doubling that of Australia (2.70 t/ha) in the same reporting period. Generally, it can be concluded that the sorghum production and yield are rising steadily in the non-traditionally growing countries, suggesting increased demand, and use of technology in its production.

2.8 Breeding of sorghum

As has already been elucidated, sorghum productivity is constrained by different biotic and abiotic factors, key among them being drought and *striga* (a parasitic weed). The need to avail varieties that are tolerant and resistant can therefore not be over-emphasized. In addition, as a result of utilization of sorghum as food and for industrial processing, certain specific grain qualities such as grain colour and size are important for farmers and the market (Basavaraj et al. 2015), providing more reasons for sorghum improvement through breeding. For instance, as Mindaye et al. (2016) explain, increased grain size with corneous endosperm is preferred as larger seeded varieties fetch a better price possibly due to higher milling yields and higher water absorbance. Further, Mekbib (2006) pointed out that sorghum stover which has uses for feed, fuel and construction of fences is often valued as highly as grain yield, hence taller varieties are highly preferred by farmers, another objective for many breeding programs.

The demand for improved varieties with both higher grain yield and farmer's preferred traits is increasing due to the rapidly growing human population and changing standards of living (Taiz 2013). According to Mindaye et al. (2016), hybrid technology could have the potential to increase productivity while retaining high biomass and large grain size. Sorghum hybrids have been grown by farmers in developed countries since the late 1950s after the discovery of a viable cytoplasmic male sterility system, allowing cost-effective hybrid production, and are increasingly being adopted in the developing world (Mindaye et al. 2016). Duvick (2015) opines that in sorghum, superiority of the F_1, or hybrid vigour, can result in a 30–40% increase in grain yield, depending on the environment and the genotypes used. Mindaye et al. (2016) offers that in addition to increasing yield, sorghum hybrid vigour has also been demonstrated to have increased yield stability over inbred lines, particularly in stressed environments.

Research aimed at enhancing sorghum resistance to stress factors should be pursued to expand the range of its growth to marginal and barren soils to meet the needs of a growing population, changing diets, and biofuel production (Anami et al. 2015); and assure its availability as food in drought prone environments in the tropics and subtropics (Srinivasa-Rao et al. 2014). It can tolerate low unpredictable rainfall, being a drought resistant crop (Adhikari et al. 2015). Despite remarkable success, sorghum research could yield fruits in a shorter period, unlike the current trend, where, according to the data presented by Ndjeunga et al. (2015), there had only been a release of 131 new varieties in the period 1970–2010, in five countries namely Burkina-Faso, Mali, Niger, Nigeria and Senegal. The challenge of the time of genotype development coupled with a more efficient screening of grain and the superior crop trait adoption can be achieved through the biotechnology-tissue culture- tool of doubled haploid technology (Barkley and Chumley, 2012). Knowledge of the cell cycle as well as gametogenesis is necessary for the exploitation of tissue culture techniques.

2.9 Cell cycle

The cell division cycle, or the cell cycle is the process by which cells reproduce themselves and their genetic material (Wilson 1925), the nuclear DNA. The cell cycle consists of four phases: G_1, S, G_2 and M (Mitchison 1971). G_1 is the phase when a newly formed daughter cell has not yet replicated its DNA (Barberis et al. 2007). The DNA is replicated during the S phase (Howard and Pelc, 1953). Further, G_2 is the phase when a cell with replicate DNA has not yet proceeded to mitosis. Collectively, G_1, S and G_2 are referred to as interphase. The M phase is the phase in which mitosis and meiosis occur. While mitosis results in cell division for the somatic cells, meiosis is the process of cell division in gametogenesis, where pollen cells that are essential for doubled haploid technology are formed.

2.10 Development of the male gametophyte

Flowering plants maintain various populations of undifferentiated stem cells mostly in meristems, the meristematic tissue of which is capable of growth and differentiation to form vegetative tissues and organs, eventually giving rise to reproductive organs containing diploid sporogenous cells (Gross-Hardt and Laux, 2003). As explained by Borg et al. (2009), a strict male germline is only established after meiosis when haploid microspores divide asymmetrically to form a small germ cell and a large vegetative cell. Rather it undergoes a single round of mitosis to produce the functional twin sperm cells required for double fertilization (Borg et al. 2009). According to Scott et al. (2004), the development of the stamen (male gametophyte) in flowering plants takes place within specialized male reproductive organs called the stamens and consists of two distinct

sequential phases, microsporogenesis and microgametogenesis. Scott et al. (2004) further explains that during microsporogenesis, diploid pollen mother cells undergo meiotic division to produce tetrads of haploid microspores. This stage is completed when distinct unicellular microspores are released from the tetrad by the activity of a mixture of enzymes secreted by the tapetum, the inner nutritive layer of the stamen (Scott et al. 2004).

Studies by Owen and Makaroff (1995) and Yamamoto et al. (2003) expound that during microgametogenesis, the released microspores enlarge, and a single large vacuole is produced. Borg et al. (2009) explain that the vacuole enlargement is accompanied by migration of the microspore nucleus to a peripheral position against the cell wall, which then undergoes an asymmetric cell division known as Pollen Mitosis I (PMI). The small germ cell, representing the male germline, is subsequently engulfed within the cytoplasm of the larger vegetative cell to create a novel cell-within-a-cell structure (Borg et al. 2009).

As explained by Borg et al. (2009), the engulfing process involves degradation of the hemispherical callose wall that separates the newly formed vegetative and germ cells (Fig. 2). Studies (Palevitz and Cresti, 1989; Cai and Cresti, 2006) explain that the fully engulfed germ cell forms a spindle-like shape that is maintained by a cortical cage of bundled microtubules. According to Twell et al. (1998), the asymmetric division at PMI is essential for the correct cellular patterning of the male gametophyte, since the resulting two daughter cells each harbour a distinct cytoplasm and possess unique gene expression profiles that confer their distinct structures and cell fates. Eady et al. (1995) elucidates that the induction of symmetrical division at PMI has demonstrated that vegetative cell gene expression is the default developmental pathway, and that division asymmetry is critical for correct germ cell differentiation.

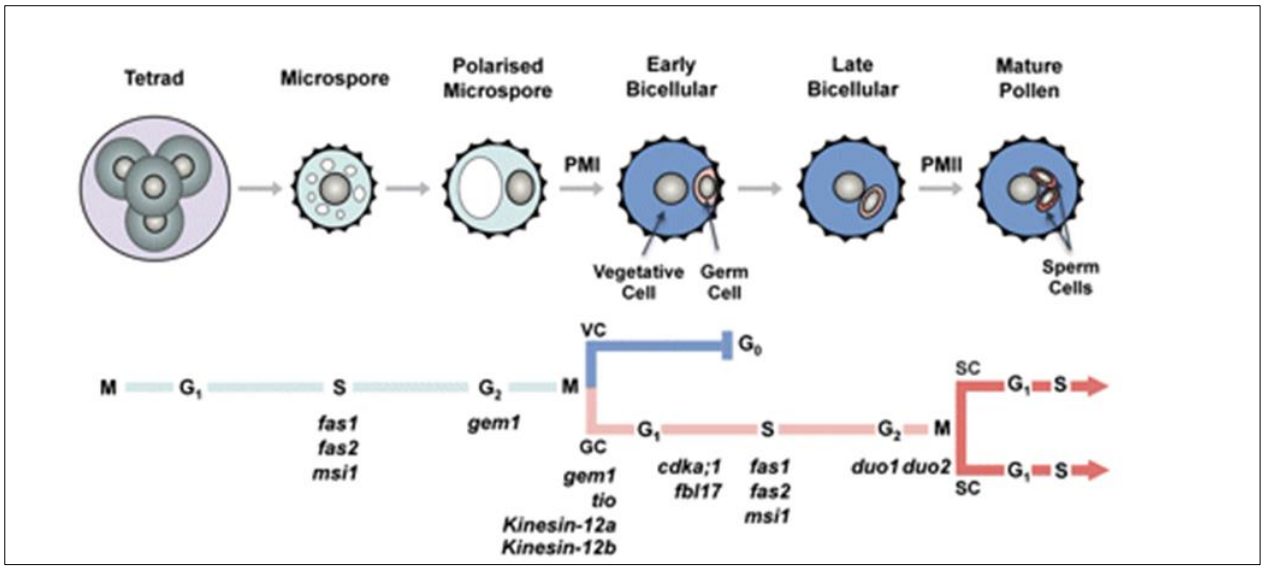

Figure 2. Schematic diagram representing the distinct morphological stages of male gametophyte

development along with a colour-coded timeline of the cell cycle progression of each cell type (adapted from Borg et al. 2009). VC, vegetative cell; GC, germ cell; SC, sperm cell.

As Borg et al. (2009) explain, following PMI, the large vegetative cell has dispersed nuclear chromatin and exits the cell cycle in G_1. The vegetative cell nurtures the developing germ cell and gives rise to the pollen tube following successful pollination, which grows through the stylar tissues of the gynoecium to deliver twin sperm cells to the embryo sac (Borg et al. 2009). According to Pacini (1996) in the process of pollen maturation, the vegetative cell accumulates carbohydrate and/or lipid reserves along with transcripts and proteins that are required for rapid pollen tube growth.

Schwacke et al. (1999) point out that osmoprotectants, including disaccharides, proline and glycine-betaine, which are thought to protect vital membranes and proteins from damage during dehydration, are also accumulated during the pollen maturation. Following the pollen maturation, as explained by Borg et al. (2009), the smaller germ cell has condensed nuclear chromatin and continues through a further round of mitosis, called Pollen Mitosis II (PMII), to produce twin sperm cells. A physical association between the sperm cells and the vegetative nucleus that is referred to as the male germ unit (MGU) is then established, following PMII (Borg et al. 2009).

2.11 Plant cell and tissue cultures

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions often to produce the clones of plants (Hussain et al. 2012). Further, Hussain et al. (2012) elucidates that the resultant clones are true-to type of the selected genotype. According to Hussain et al. (2012), the technique depends mainly on the concept of totipotency of plant cells which refers to the ability of a single cell to express the full genome by cell division. As Fehér (2019) opines, in addition to the totipotency of plant cells, the capacity of cells to alter their metabolism, growth and development is also equally important and crucial to regenerate the entire plant. According to Hussain et al. (2012), the controlled conditions provide the culture an environment conducive for cell growth and multiplication. Hussain et al. (2012) enumerates these conditions as adequate supply of nutrients, pH of medium, adequate temperature and proper gaseous and liquid environment, which are realized using an appropriate culture medium.

As Saad and Elshahed (2012) explain, an appropriate tissue culture medium contains all the nutrients required for the normal growth and development of plants and is mainly composed of

macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium. Sandra et al. (2016) suggested that pH alongside plant growth regulators are important in the plant growth and the control of antioxidant activity on the medium. As exemplified by Mbiyu et al. (2012), both the solid and liquid medium can be used for culturing. The composition of the medium, particularly the plant hormones and the nitrogen source has profound effects on the response of the initial explant (Hussain et al. 2012).

According to Rahmat and Kang (2019), plant growth regulators (PGR's) play an essential role in determining the development pathway of plant cells and tissues in culture medium. Gaspar et al. (1996) lists some of the main plant growth regulators as auxins, cytokinins and gibberellins. The type and the concentration of hormones used depend mainly on the species of the plant, the tissue or organ cultured and the objective of the experiment (Hussain et al. 2012). According to Hussain et al. (2012), auxins and cytokinins are most widely used plant growth regulators in plant tissue culture and their amount determine the type of culture established or regenerated.

Skoog and Miller (1957) offer that elevated concentration of auxins generally favours root development, whereas the high concentration of cytokinins promotes shoot regeneration. As explained by Ikeuchi et al. (2013), a balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus. Gibberellins play a critical role in cell elongation and plant growth (Sun 2010).

2.11.1 Sorghum tissue cultures

Sorghum is one of the most difficult crops for tissue cultures. The success has been restricted by low callus induction rate, low regeneration rate, exudation of pigments or phenolic compounds from somatic cells and albino plantlets (Liang et al. 1997). Several studies have described calli initiation protocols for sorghum although with little reproducibility. While it has been reported that the success of sorghum embryogenesis is genotype dependent, various studies have reported varying degrees of callus formation influenced by different modifications of culture media (Teingtham 2017). The explant type has also been shown to have a great role in minimizing phenolics and increasing the regeneration rates (Liu et al. 2015).

Some protocols have been established for explants such as immature embryos (Nguyen et al. 2007; Grootboom et al. 2008; Muhumuza and Okori, 2013), immature inflorescences (Zarif et al. 2013), shoot tips (Seetharama et al. 2000; Kingsley and Ignacimuthu, 2014), leaf base (Mishra and Khurana, 2003), leaf segments (Pola and Mani, 2006) and also from cultured mesophyll

protoplasts (Sairam et al. 1999). However, the frequency of plant regeneration reported so far does not seem to be high enough (Assem et al. 2014). An efficient tissue culture protocol for sorghum like in other crops would enable acquisition of downstream techniques that depend on regeneration for gene delivery (Sadia et al. 2010), such as gene transformation ((Liu et al. 2015). and genome editing.

2.11.2 Doubled haploid production in sorghum

Doubled haploid lines in sorghum can reduce the time to homozygosity in breeding by accelerating efficacy in selection and eliminating the need for prolonged breeding cycles (Chang and Coe, 2009; Wędzony et al. 2009). The technology's development has involved many researchers over a long period as explained by Wędzony et al. (2009). It requires acquisition of haploids firstly, currently achieved through *in vitro* androgenesis and gynogenesis sporophytic development induction, wide hybridization and sometimes obtained spontaneously although at a very low frequency in almost every plant species (Hussain and Franks, 2019).

Androgenesis can be achieved through anther culture or by isolated microspore culture depending on the method followed, with protocols now existing for more than 200 species (Wędzony et al. 2009; Bhojwani and Dantu, 2013). The process leads to the development of embryos rather than pollen grains from male gametes thus requiring a complete reprogramming of the developmental plan. The induction sees to the shift from maturation of the highly specialized pollen cells to sporophytic development and is only possible, as Touraev et al. (2001) opines at very early developmental stages when the gametic cells appear to be totipotent.

The technology draws from the fact that in certain conditions, the microspores which are the first generation of male gametic cells and that have a haploid chromosome complement are amenable to androgenic induction in many species, their production in plant anthers in large numbers providing an advantage as they are relatively easy to access and manipulate (Touraev et al. 2001; Wędzony et al. 2009). These conditions include but are not limited to anther, inflorescence and/or donor tiller temperature shock; starvation; and/or other stress treatment (Žárský et al. 1992, 1995; Touraev et al. 1996); medium composition; and growth regulators (Wędzony et al. 2009). In gynogenesis, reprogramming is not necessary as the embryogenic pathway just needs to be switched on in the female gamete without proper fertilization (Portemer et al. 2015).

Wędzony et al. (2009) argues that compared to androgenesis, gynogenesis is not effective because of the far fewer eggs produced by plantlets than pollen grains. Nevertheless, haploids induced from unfertilized ovule and ovary cultures have been reported for many plant species such as *Beta*

vulgaris L., *Allium cepa* L. and others that are recalcitrant to androgenesis (Keller and Korzun, 1996; Teingtham 2017).

Wide hybridization, a technique where a genetically relatively distant male partner or pollinator possessing special genetic properties such as haploid inducing genes is crossed with a desirable female has been reported to be effective in haploid production (Hussain and Franks, 2019). On fertilization, the male genetic material gets eliminated from cells of the developing embryo at early stages of its growth (Wędzony et al. 2009). According to Hussain and Franks (2019), when an inducer line is used to pollinate a diploid plant, most of the embryos produced are regular hybrid embryos, but a smaller proportion of haploid maternal embryos are produced.

2.11.3 Progress in doubled haploid development in sorghum.

The benefits of doubled haploid like in other major cereals can immensely impact sorghum hybrid development. As Wędzony et al. (2009) explains, progress in the doubled haploid technology has been achieved through empirical, time- and cost-consuming testing of protocols, and consequently, success over the last few decades has been proportional to the number of laboratories involved. The detailed review by Wędzony et al. (2009) mentions the frequently studied crops whose protocols for the technology have now become routine as barley, triticale, maize, rice and rapeseed.

Even with the immense and urgent need to accelerate sorghum hybrid development, it is conspicuously missing out on this list and the cumulative benefits thereof. This is despite its strategic advantage as the grain of the future, due to its low gluten content, high lipid content (Pontieri et al. 2013), in addition to tolerance to climate change related abiotic stresses such as drought. Some authors point out that the doubled haploid protocols for sorghum remain unavailable or unreproducible (Teingtham 2017, Chege et al. 2020a). This is not entirely unexpected as sorghum has been described as one of the most recalcitrant crops for tissue cultures (Zhao et al. 2000). Liang et al. (1997) opined that factors such as low rate of callus induction and regeneration, high level of poly-phenols exudation from somatic tissues and appearance of albino plantlets among regenerants contribute to the dismal success of tissue cultures in sorghum.

In somatic tissue culture, Liu et al. (2015) argued that success of sorghum embryogenesis is genotype dependent, although factors such as the explant type and different culture medium modifications have played a role in successful callus formation at various degrees (Teingtham 2017). Most significantly, in addition to their findings that explant type plays a great role in suppressing polyphenols production and contributing to increased regeneration rates, Liu et al.

(2015) reported a drastic reduction in polyphenols production after their induction medium modification with amino acids L- proline and L- asparagine, together with KH_2PO_4 and $CuSO_4.5H_2O$ to MS medium (Murashige and Skoog, 1962). Further to this work, Chege et al. (2020a) reported that the use of half-strength MS medium together with the supplements used by Liu et al. (2015) contributed to attainment of quality callus i.e., 60% yellow calli with 25% of them being friable.

A doubled haploid development protocol may, however, not benefit from all the gains made in somatic embryogenesis, especially during callus induction, owing to the difference in the explant types involved. Notable progress has been realized in sorghum doubled haploid production following recent findings in the work of Hussain and Franks (2019) where they report sorghum cultivars SMHI01 and SMHI02 as putative haploid inducer lines, although their success rates are very low (1–2%). A patent has been sought for a genetic modification of an endogenous gene encoding patatin phospholipase therefore conferring haploid inducer ability or enhancing the induction performance of sorghum plants <https://patentimages.storage.googleapis.com-/64/fd/ef/5b5c09747d87c2/EP3366778A1.pdf>.

These two haploid inducer development methods may be faced with a common problem — early desiccation of embryos from mutants and wide hybridization — as described by Rizal et al. (2014). A method for speed breeding developed by Rizal et al. (2014) in which by splitting a panicle on a single culm, both selfed and crossed progenies were obtained and through embryo rescue the breeding cycle was reduced from 17 to 11 weeks, is a step forward in addressing the mentioned challenges. Some of the other speed breeding techniques described by Hickey et al. (2019) may be explored, to augment, complement or wholly replace the need for doubled haploid technology in future.

2.11.4 Androgenesis-based tissue culture methods in sorghum

Research geared towards the optimization of a doubled haploid development protocol for sorghum by Rose et al. (1986) represents one of the earliest works in the crop. They reported the importance of incubation temperature in anther response rate and 33 °C as the optimum, although they regenerated only four albino plantlets of unknown ploidy from more than 1,000 anther-origin calli. In what was a significant improvement from these findings, Wen et al. (1991) regenerated 29 plantlets from one cultivar ‘Xin White’, on medium MS-d-4 (modified Murashige and Skoog, [1962] medium) out of an anther culture of six sorghum cultivars on three induction media.

As a result of the albino haploids among the regenerants, Wen et al. (1991) concluded that they may have developed sorghum haploids. They, however, at the same time identified diploid number of chromosomes in some of the regenerants, which pointed out that some of the induced calli may have been from somatic tissues rather than from microspores. Like many other works involving sorghum tissue culture, the callus induction frequencies in this work being 4.3%, 3.2% and 0.1% for media C17-2, MS-t-z-2 and 85D3-2, respectively were low, and genotype dependence was observed (Wen et al. 1991).

In a later experiment, Kumaravadivel and Sree Rangasamy (1994) successfully induced calli at the rate of 40 calli per every 100 anthers in a culture of a hybrid line of sorghum, CSH5 (2077A × C83541). The anthers of the hybrid line had been cultured on N6 medium (Chu et al. 1975) supplemented with 3.0% sucrose, 2.0 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.8% agar incubated at 30 °C. The experiment reported a regeneration frequency of 7.6% on modified MS medium supplemented with 3.0% sucrose, 2.0 mg/BAP combined with 0.3 mg/1 indole-acetic acid (IAA) and for gelatinizing the medium, 0.8% agar was used (Kumaravadivel and Sree Rangasamy, 1994).

2.11.4.1 Pre-treatment of donor tillers for sorghum

As mentioned earlier, for androgenic induction, certain conditions (abiotic stresses) must be met for the male gametes to be reprogrammed to sporophytic development. While Wędzony et al. (2009) argued that the role of abiotic stress factors in androgenic induction are not completely understood, and therefore not perfectly controlled in most experiments, still, they provided case for two distinct functions in their review: synchronization of the developmental process of microspores (Hu and Kasha, 1999) and determination of the developmental stage of microspores (Binarova et al. 1993; Hause et al. 1993).

Some of the stresses as earlier stipulated and as presented by Bhojwani and Dantu (2013) include: starvation, temperature shock, osmotic stress, colchicine, n-butanol treatment and others applied to whole plants *in vivo*, tillers, buds, or isolated anthers or microspore *in vitro*. In their work, Rose et al. (1986) pre-treated sorghum tillers by wrapping them in polyethylene and aluminium foil and keeping them in darkness for 28, 35 or 42 days at 7°C; 10, 14 or 18 days at 14°C; 7, 10 or 13 days at 20°C; 3, 4 or 5 days at 25°C; and 1, 2, 3 or 4 days at 28°C. They further included an unpretreated control and distributed tillers sequentially among the treatments with seven repeats over the growing period to avoid the effect of donor plant environment on anther performance *in vitro*.

At the ideal incubation temperature (33 °C), Rose et al. (1986) concluded that long-temperature stress pre-treatments were not advantageous to callus induction. They argued that panicle pre-treatments at 7, 14, 20, 25 and 28 °C either had no effect or significantly decreased callus yield.

The experiment rather, found incubation temperature to have significant effect on yields of microspore-derived callus. On their part, Wen et al. (1991) did not conduct any pre-treatments other than panicle sterilization in 60% ethanol, which as Wędzony et al. (2009) argue, could be considered a stress treatment. According to Wen et al. (1991), callus induction was genotype dependent for any given medium and culture conditions. In the work of Kumaravadivel and Sree Rangasamy (1994), a heat shock of 35 °C for 12 hr followed by an incubation temperature of 25 °C was included in one of the treatments, however, it did not prove to have any advantage to the eventual number of calli induced.

Ideal incubation temperature was argued to be the main causative of callus induction (Kumaravadivel and Sree Rangasamy, 1994). This argument corroborated that of Rose et al. (1986), although they differed in the ideal incubation temperature as already pointed out earlier. Apart from temperature stress, whose role in influencing microspore transition from gametophytic to sporophytic development was discussed by Touraev et al. (1997), no work on sorghum anther culture has been done with other types of pre-treatment.

In recent works, it is now quite commonplace to combine a relatively short low or high temperature treatment with osmotic starvation stress or interchange the two (Hoekstra et al. 1997; Kasha et al. 2002; Jacquard et al. 2003; Wędzony et al. 2009), such as a pre-treatment of 14°C in a medium containing mannitol, that was done to improve androgenic efficiency in maize (Nageli et al. 1999; Zheng et al. 2003). In a separate experiment, when used together with growth regulators, starvation stress in mannitol gave satisfactory results in callus induction in maize (Liu et al. 2002a, 2002b). In an experiment on cytology of barley (*Hordeum vulgare* L.) microspores at uni-nucleate stage during pre-treatment at the early culture stage, Kasha et al. (2001) concluded that spike pre-treatment with 0.3 M mannitol at 4 °C for 4 days was preferable as it appeared to provide genotype independent induction and suspension of nuclear division as well as regeneration of green plants at a shorter time compared to the case when the spikes were pre-treated with cold temperature alone, findings that are similar to those of Liu et al. (2002a).

Prior to this, Indrianto et al. (1999) had demonstrated that wheat microspore embryogenesis was possible with a combination of high temperature shock in mannitol treatment. Zhao et al. (1996) had demonstrated that colchicine could induce microspore embryogenesis without requiring heat

shock (at non-inductive temperature of 25 °C) in *Brassica napus* L. cv. 'Topas'. The above examples go to reiterate that stress is the major trigger in inducing microspore embryogenesis from many monocots and dicots as pointed out by Touraev et al. (1997).

Many of the existing protocols utilize one or more of the common stress inducing components: heat shock, cold shock, carbohydrate or nitrogen starvation and colchicine pre-treatment (Indrianto et al. 1999), but besides the work of Rose et al. (1986), literature for pre-treatment in sorghum anther culture is largely lacking. Rose et al. (1986) opined that inductive benefits of pre-treatment maybe partly offset by the acceleration of production of polyphenols in the anther wall in sorghum. According to them, tissue damage in sorghum tend to stimulate production of polyphenols, also referred to by some authors as lethal browning, therefore causing death to the developing microspores (Ko et al. 2008).

2.11.4.2 Culture media and conditions utilized to induce sorghum androgenesis

Like other androgenic inductions, sorghum anther culture would require a culture medium with an organic nitrogen source, carbohydrates and growth regulator components as stipulated by Wędzonymet al. (2009). Unfortunately for the crop, there has not been a reproducible culture medium. Rose at al. (1986) reported a total of 1,174 anther-derived calli induced on liquid potato extract (Chuang et al. 1978) with 9% sucrose (w/v), 0.5 mg/L kinetin, and different concentrations of 2,4-D incubated in darkness at 33 °C. The callus yield and anther response rates were highest on the medium containing 2–3 mg/L 2,4-D concentrations and that there was a marked difference in phenolics production (medium discolouration) between the medium that had 2,4-D and those without.

On regeneration, this first sorghum anther culture experiment utilized half strength MS medium (Murashige and Skoog, 1962), modified with a range of auxins and cytokinins at different concentrations. They used kinetin at 0.5 mg/L which had been reported by Wernicke et al. (1982) to stimulate organ differentiation from leaf cultures of sorghum; and α-naphthalene-acetic acid (NAA) at 5 mg/L which Masteller and Holden (1970) had recommended for sorghum callus differentiation. Rose et al. (1986) additionally used a combination of BAP at 2 mg/L and IAA at 1 mg/L as had been used by Huang and Sunderland (1982) for barley anther-derived callus differentiation.

The regeneration media used in this experiment also comprised of 2% (w/v) sucrose and 0.8% (w/v) agar as the gelling agent. It was reported that incubation temperature had a significant effect on anther response, with those incubated at 33 °C in a 10 hr-photoperiod giving the

highest response and those incubated at 20, 25 or 30 °C rapidly turned black and died (Rose et al. 1986). At this temperature, they also reported a reduced discolouration of the medium and anthers. Regeneration entailed 3 stages. In the first stage, calli were transferred to a regeneration medium containing 2 mg/L 2,4-D. This resulted in individual calli proliferation, where they lost their original structure.

The basal calli that were contact with the medium had a yellow colour, were relatively loose and unstructured. The surface cells on the other hand either formed smooth brown gelatinous callus or compact white partially structured calli, which were sub-cultured every 7–10 days failure to which the calli discoloured. The second stage involved the transfer of the semi structured basal calli from the first stage onto a medium containing BAP and 2 mg/L 2,4-D, which were then transferred to the third stage after 14 days. In the final stage, four calli were regenerated into albino plantlets on a half strength MS medium containing only 0.1 mg/L 2,4-D.

In their work, Wen et al. (1991) incubated 21,000 anthers over a period of 18 months on three media (Tab. 1) namely MS-t-z-2, C17-2, and 85D3-2 in darkness at 28±°C until calli were formed. Out of the 29 varieties involved in the trial, only six namely 'Xin White', 'Tx 403-TSB', 'DDY Sooner milo', 'Tx 2779', 'Brawley', and 'Spur Federal' formed calli at different frequencies, with 'Xin White' and 'TX 403-TSB' having significantly higher callus induction frequencies than the other four at 6.7% and 3.9%, respectively. There were significantly higher callus induction frequency rates on media C17-2 (3.6%) and MS-t-z-2 (4.1%) than on medium 85D3-2 (0.1%). Wen et al. (1991) concluded that addition of myo-inositol in MS-t-z-2 and biotin in C17-2 appeared to be beneficial to callus induction and that compared to NAA, 2,4-D as the auxin provided better results.

The sorghum calli induced in the work of Wen et al. (1991) were regenerated on medium MS-d-4 (MS medium supplemented with 2 mg/L IAA and 2.5 mg/L kinetin). Shoots began regenerating on the 3 mm calli that were transferred to the regeneration media two to three weeks later. Wen et al. (1991) observed that shoots formed before roots in normal regeneration while calli that developed roots first never developed shoots. They also reported that the more the callus induction time, the less the callus regeneration frequency was, and it led to increased number of albino plantlets or regeneration of only roots in a callus.

The experiment by Kumaravadivel and Sree Rangasamy (1994) involved the culture of anthers of a sorghum hybrid CSH5 (2077A x C83541) on N6 medium (Chu et al. 1975) modified with 2.0 mg/L 2,4-D, 3.0% (w/v) sucrose and 0.8% (w/v) agar at pH 5.8. In the first part of the trial, the anthers were incubated in the dark at 15, 20, 25, 30 and 35 °C. A total of one hundred and twenty

anthers were cultured in each treatment. The effect of different concentrations of 2,4-D at 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L in the induction media were tested in the second part of the experiment where the same number of anthers per treatment were incubated in the dark, at 30° C and 85% relative humidity in media with the same components as in part one, but with the different 2,4-D concentrations (Kumaravadivel and Sree Rangasamy, 1994).

In the first part, 30 °C was reported to have the highest callus induction frequency at 40% as shown in Tab. 2. In the second part of the experiment, 2,4-D at 2 mg/L was shown to be the most effective in callus induction (Fig. 3). It was reported that anthers turned brown 7–10 days after incubation, but white to yellow compact and nodular calli began to grow by the 15th day of subculture and proliferated on culture for 25–30 days (Kumaravadivel and Sree Rangasamy, 1994).

Table 1. Components of the media used by Wen et al. (1991) for sorghum anther culture callus induction and regeneration medium 190-2 Cu by Pauk et al. (2003).

Components	**Media (mg/l)**			
	MS-t-z-2	**C17-2**	**85D3-2**	**190-2Cu**
KNO_3	1900	1400	2830	1000
KH_2PO_4	170	400	400	300
NH_4NO_3	1650	300	-	-
$(NH_4)_2SO_4$	-	-	-	200
$CaCl_2x2H_2O$	440	150	166	-
$MgSO_4x7H_2O$	370	150	185	200
$Ca(NO_3)_2x4H_2O$	-	-	-	100
or $MgSO_4$	180	73.2	90.2	-
KCl				40
Na_2.EDTA	37.3	37.3	37.3	37.3
$FeSO_4x7H_2O$	27.8	27.8	27.8	27.8
$MnSO_4x4H_2O$	22.3	11.2	4.4	8
(or $MnSO_4xH_2O$)	17.1	8.6	3.4	-
$ZnSO_4x7H_2O$	8.6	8.6	1.5	3
H_3BO_3	6.2	6.2	1.6	3
KI	0.83	0.83	0.8	0.5
$CuSO_4$ x $5H_2O$	0.025	0.025	-	0.5
$CoCl_2$ x$6H_2O$	0.05	0.025	-	-
$NaMoO_4$ x $2H_2O$	0.25	-	-	-
Glycine	2	2	2	2
Thiamine HCl	0.1	1	1	1
Pyridoxine HCl	0.5	0.5	0.5	0.5

Nicotinic acid	0.5	0.5	0.5	0.5
Myo-inositol	100	-	-	100
Casein Hydrolysate	-	-	500	-
Biotin	-	1.5	-	-
2,4-D	3	2	-	-
NAA	-	-	1	0.5
Kinetin	0.3	1	1.5	0.5
Zeatin	2.2	-	-	
Sucrose	20,000	20,000	30,000	30,000
Difco agar	5,000	7,000	5,000	-
Gelrite				2,800
pH	5.8	5.8	5.8	5.8

Table 2. Effect of incubation temperature on callus induction on day 25 as reported by Kumaravadivel and Sree Rangasamy (1994)

Incubation Temperature (˚C)					
	15	20	25	30	35*
No. of anthers with calli (%)	10.1 ± 0.8	16.4 ± 2.2	23.7 ± 1.5	27.6 ± 2.1	20.5 ± 3.0
No. of calli/100 anthers	12.3 ± 1.0	20.0 ± 0.7	27.1 ± 3.0	39.9 ± 1.0	23.0 ± 2.1
*35˚C for 12 hrs followed by incubation at 25˚C					

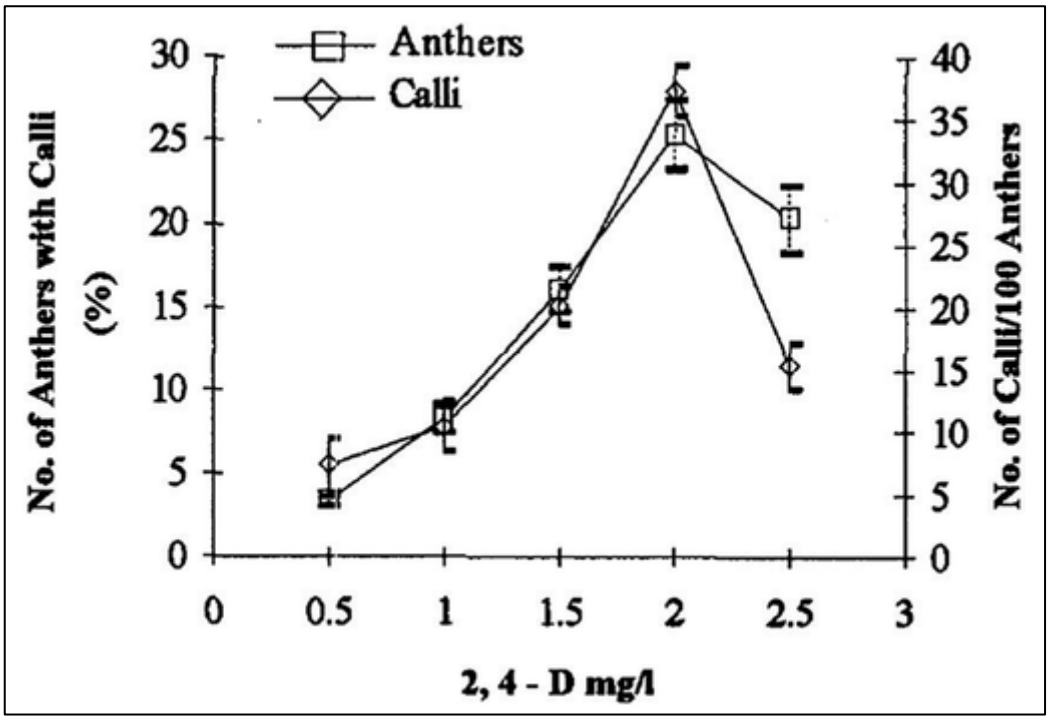

Figure 3. The effect of 2,4-D on sorghum callus induction at 30 ˚C on day 25 in anther culture (adapted from Kumaravadivel and Sree Rangasamy, 1994)

Kumaravadivel and Sree Rangasamy (1994) used MS medium modified with B5 vitamins (Gamborg et al. 1968), FeEDTA in double quantity with concentrations of BAP at 1.5, 2.0 and 2.5 mg/L and IAA at 0.3 mg/L to determine the optimum concentration of BAP and IAA necessary for sorghum plantlets regeneration. The embryogenic calli were transferred to the media and incubated at 25±2°C under a 16-hr photoperiod. They reported that on the regeneration media, calli formed clusters of embryoids and contrary to the findings of Wen et al. (1991), roots and shoots developed simultaneously. Regeneration media comprising of 2.0 mg/L BAP and 0.3 IAA gave the highest calli yield. A total of 1,417 plants were regenerated, whereby 764 were green and 653 were albinos (Kumaravadivel and Sree Rangasamy, 1994).

In addition to these three studies, Can et al. (1998) induced sorghum calli from six cultivars at a mean frequency of 5.1%. MS medium containing 2.0 mg/L kinetin, 1.0 mg/L IAA and 2.5 mg/L 2,4-D produced the highest callus induction frequency at 6.4% with the field grown anthers and 3.7% with the greenhouse grown anthers after incubation in darkness at 30 ± 1°C, where a total of 12,000 anthers were incubated. Can et al. (1998) found MS medium modified with 2.5 mg/L kinetin and 3.0 mg/L IAA to be the most effective for regeneration resulting in 27% frequency. A total of 63 plantlets were regenerated from the 248 calli obtained, out of which 43 were green and 20 albinos (Can et al. 1998).

2.11.5 Ploidy level determination and chromosome duplication

According to Dunwell (2010), haploids can be distinguished from their diploid counterparts by their generally small appearance owing to their smaller cell size as cell volume in plants is directly proportional to their ploidy level. However, there are various methods through which the ploidy level can be confirmed. Cytological techniques and measurement of the DNA using flow cytometry are the direct methods of ploidy level determination while the use of guard cell and plastid dimensions comprise the indirect methods (Yuan et al. 2009; Dunwell, 2010). Methods utilizing DNA markers for ploidy determination have also been described (Diao et al. 2009).

In the sorghum anther culture induction work of Rose et al. (1986), the ploidy level of the albino regenerants was neither determined nor discussed. Wen et al. (1991) on the other hand examined root tips from microspore derived calli using the method described by Tang and Liang (1987). There were 10, 20 and 40 chromosomes counted from root-tip cells of the regenerated plants of Xin White variety (Wen et al. 1991). They reasoned that occurrence of 20 chromosomes from anther culture indicated that calli may have originated from somatic cells rather than from microspores. However, they argued that regeneration of albino plantlets suggested haploid plants may have been regenerated and that a few of the albino plantlets could have been a result of spontaneous chromosome doubling. Chromosome doubling was not reported for this work.

Twelve haploid and 248 doubled haploid plants were reported to have been regenerated in the work of Kumaravadivel and Sree Rangasamy (1994) from the culture of anthers from hybrid variety CSH5 (2077A x C83541). Similar to the work of Wen et al. (1991), chromosome counting of root tip cells was the method employed to determine ploidy level (Kumaravadivel and Sree Rangasamy, 1994). It was reported that all the haploids developed and 116 of the 2n plants were sterile while the rest were fertile. Sterility in the 2n plants was thought to have been brought about by sterility inducing (S) cytoplasm from one of the parents (2077A) of the hybrid (Kumaravadivel and Sree Rangasamy, 1994).

Spontaneous chromosome doubling was reported to have occurred in 95.3% of the regenerated haploids, and therefore Kumaravadivel and Sree Rangasamy (1994) deemed genome doubling procedures to be unnecessary. On conducting field experiments that compared the regenerants with the original and selfed hybrid, it was observed that both quantitative and qualitative traits' variances were non-significant within family groups of the regenerants indicating rapid attainment of homozygosity, but significantly different when compared to those of the hybrid (Kumaravadivel and Sree Rangasamy, 1994). According to Kumaravadivel and Sree Rangasamy (1994), the field performance of the regenerants strongly suggested the gametophytic origin of the explant and

indicated fixation of alleles as expected in doubled haploids. In their work, Can et al. (1998) observed 20 chromosomes (2n) in most of the regenerants and unstable chromosome numbers in the rest. Can et al. (1998) used the method described by Hinata (1990) and modified by Can and Yoshida (1997) in determining ploidy level by root tip cell chromosomes observation. They concluded that the diploid plants observed may have been a result of spontaneous chromosome doubling of haploids or due to culture of somatic cells (Can et al. 1998). The summary of the progress in development of sorghum doubled haploids can be visualized in Tab. 3.

Table 3. Summary of the progress in sorghum doubled haploids dvelopment

Publication	**No. of genotypes involved**	**No. of Calli induced**	**No. of regenerants**	**No. of DH lines produced**
Rose et al. (1986)	1 genotype	1174	4 albino plantlets	Unknown ploidy
Wen et al. (1991)	29 genotypes	3.5 per 100 anthers	29 (including albino plantlets)	Not determined
Kumaravadivel and Sree Rangasamy (1994)	1 genotype	40 per 100 anthers	1417 (764 green, 653 albinos)	12 haploid, 248 DH lines
Can et al. (1998)	6 genotypes	5.1 per 100 anthers	63 plantlets (43 green, 20 albinos)	Most regenerants; some had unstable chromosomes

2.12 Genotypic diversity analysis in sorghum

Despite being useful for crop breeders, the challenge of agronomical and morphological characterization is that phenotypic characters are influenced by environmental factors (Govindaraj et al. 2015). The diversity analysis based alone on morphological features remains inefficient due to the number of constraints such as stage and tissue specific expression of the traits, influence of environment, existence of pleiotropy among others (Smith and Smith, 1992). Therefore, genotypic characterisation is dependent upon use of molecular marker systems because these are not under the influence of environmental conditions (Meszaros et al. 2007). They also do not require fixed plant developmental stages and have a potential to rapidly give results from both seeds and seedlings. Hence, molecular markers, in combination with morphological and / or biochemical markers, along with pedigree information can be efficiently utilized for better assessment of genetic diversity in sorghum (Geleta et al. 2006).

2.12.1 Measurement of genetic diversity

Before the advent of molecular marker techniques, genetic diversity estimations involved different biometric techniques such as metro glyph, divergence analysis, phenotypic diversity index (H), morphological aspects by utilizing parentage, principal component analysis, agronomical and biochemical data (Mohammadi and Prasanna, 2003). This, however, was found to be time-consuming and laborious in carrying out evaluation based on phenotypic data (Rauf et al. 2009).

The discovery of different molecular markers techniques resulted in efficient estimation of genetic diversity based on data being generated by the different molecular markers. This enhanced rapid analysis of germplasm and estimation of genetic diversity which were observed to corroborate the phenotypic data. The molecular markers are independent of environmental factors and give rise to high level of polymorphism (Arif et al. 2010). Molecular markers can be used for fingerprinting techniques which are important tools for genetic identification and in determination of variation in plant breeding and for germplasm management (Jiang 2017).

2.12.2 Molecular markers in plant breeding

The potential application of polymorphic genetic markers in plant breeding could be in utilization of genetic markers to determine genetic relationships, including variety identification, protection of breeder's rights, and parentage determination. On the other hand, genetic markers may be used to identify and map loci affecting quantitative traits and to monitor these loci during introgression of selection programmes (Kamaluddin et al. 2017). They have been applied in breeding programs thus facilitating appropriate choice of parents for hybridization, mapping / tagging of gene blocks associated with economically important traits, DNA fingerprinting among others (Gupta and Varshney, 2000).

A variety of molecular markers have become available in recent years each having a different set of advantages in their application (Mohan et al. 1997). There are three broad categories of DNA-based molecular marker technologies that are currently used for the purpose of fingerprinting in plants namely:

(i) DNA hybridization-based markers also known as the 'first generation markers' e.g., RFLP (Restriction Fragment Length polymorphism).

(ii) Polymerase Chain Reaction (PCR) based markers that utilize primers of sequences that are arbitrary to that of the target genome, such as Randomly Amplified Polymorphic DNA (RAPD), they are known as second-generation markers.

(iii) Third generation markers such as the Single Nucleotide Polymorphism (SNP).

2.12.2.1 Simple Sequence Repeats (SSR) or microsatellites markers

SSRs, are stretches of DNA consisting of tandemly arranged units of 1 to 6 bp long repetitions (Thiel et al. 2003). The SSRs are multi-allelic and generally generate more information than most of the marker techniques; they are based on heterozygosity values. SSR markers are easily maintained and shared among laboratories (Maughan et al. 1995). They have a high reproducibility, co-dominance, low cost and they are highly variable regions in the plant genome (Powell et al. 1996). The SSRs serve as an ideal marker system for genetic analysis. Hence, they have wide usage in genetic diversity studies of various crops including sorghum. In plants, SSRs are much more abundant and preferentially associated with untranslated regions (UTRs) of the transcribed regions (Morgante et al. 2002).

The frequency of repeats decreases exponentially with repeat length (Katti et al. 2001). This might be since longer microsatellites exhibit higher mutation rates than shorter ones (Mconnell et al. 2007). They give a high throughput, being co-dominant markers, both homozygotes and heterozygotes can be easily identified and distinguished. Microsatellite technique is quite inexpensive, once the primers have been developed, it is repeatable, easily automated, requires small quantities of DNA, PCRs and gel runs can be multiplexed. However, their development – if no whole genomic sequences are available - can be long and expensive because of the requirement of establishing and screening genomic libraries and sequencing the clones carrying repetitive motifs.

Different types of SSRs exist: dinucleotides, trinucleotides and tetranucleotide repeats which are commonly used in molecular genetics studies (Selkoe and Toonen, 2006). Dinucleotide repeats are most abundant microsatellites which have been reported in many species and are commonly found in the non-coding regions as compared to the coding regions (Li et al. 2002). Trinucleotides are concentrated in the coding regions of the genome (Toth et al. 2000). The application of microsatellites includes genetic mapping and genome analysis (Akemi et al, 2012), gene and quantitative trait locus analysis (Hayden et al. 2004) and in marker assisted breeding. Microsatellites used in sorghum are highly reproducible and have a high resolution (Oliveira et al. 2006).

2.12.2.2 SSR markers in sorghum genetic diversity

Djè et al. (2000) used SSR markers to study the genetic diversity pattern in 25 sorghum genotypes collected from ICRISAT. Their results of cluster analysis indicated 70% of total genetic diversity

occurring among the accessions. The study indicated that microsatellite data is useful in identifying individual accessions with a high relative contribution to the overall allelic diversity of collection. Casa et al. (2005) on the other hand assayed 98 SSRs distributed throughout the genome in a panel of 104 accessions comprising 73 landraces and 31 wild sorghums, this study showed that landraces retained 86% of the diversity observed in the wild sorghum.

In the work of Smith et al. (2000), 15 microsatellite markers were utilized to survey genetic diversity in 50 genetically diverse elite sorghum inbred lines. SSR markers showed Polymorphic Information Content (PIC) in the range of 0.35-0.78 with a mean value of 0.58. Dendrogram based on Euclidean distances and UPGMA analysis showed the grouping of majority of released varieties into single cluster. In their work, Mutegi et al. (2011) analysed a total of 439 individuals comprising of 329 cultivated and 110 wild sorghum varieties using 24 microsatellite markers. The analysis revealed many SSR alleles (295), with high mean number of alleles per locus (12.3) and gene diversity of 0.88.

Cluster analysis of SSR data using Rogers' genetic distance and UPGMA, showed significant genetic diversity among the wild genotypes compared to the cultivated ones. Shehzad et al. (2009) estimated genetic diversity among 320 sorghum accessions collected from Africa and Asia using 38 SSR markers representing all 10 chromosomes of sorghum. They concluded a positive correlation between SSR based diversity and their geographical patterns of differentiation; they selected 108 accessions as diversity research set based on marker data.

3. MATERIALS AND METHODS

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

3.1.1 Germplasm collection

A total of 31 sorghum [*Sorghum bicolor* (L.) Moench] genotypes were used in the study, 15 of which were of East African origin from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT-Nairobi) and 16 Hungarian sourced from the Cereal Research Nonprofit Ltd, Hungary (Tab. 4). The genotypes were selected based on their relative importance to the breeding programs of the respective institutions and regions. 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 (Fig. 4).

Figure 4. Sorghum genotypes planted on sterile forest soil at the Institute of Genetics, Microbiology and Biotechnology at Szent Istvan University, Gödöllő, Hungary, August 2018.

They were watered appropriately until the plants were 14 days old, when two leaves from each genotype were harvested for genomic DNA extraction.

Table 4. List of sorghum genotypes involved in the genetic diversity study

S/No	Name of the genotype	Sample code	Source
1	ICSR93034	I/423	ICRISAT
2	Framida	I/402	ICRISAT
3	Seredo	I/405	ICRISAT
4	Hakika	I/407	ICRISAT
5	EC-Teso	I/412	ICRISAT
6	Mahube	1/420	ICRISAT
7	KARI Mtama 1	I/432	ICRISAT
8	Abaleshya	I/433	ICRISAT
9	IESV91054LT	I/440	ICRISAT
10	F67Q212	I/419	ICRISAT
11	Gadam el Hamam	I/417	ICRISAT
12	Cytanombe	I/438	ICRISAT
13	IESV900I5LT	1/439	ICRISAT
14	IESV91069LT	I/441	ICRISAT
15	Ndamoga	1/449	ICRISAT
16	Alföldi 1	I/3	CRC
17	GK Emese	I/1	CRC
18	GK Zsófia	I/2	CRC
19	SREZA× (A119 × SZc 2282)	1/7	CRC
20	ARET×VSZ21KKD	1/5	CRC
21	AREL×VSZ21KKD	I/11	CRC
22	A119 × SZeTC73	I/10	CRC
23	Farmusugro 180	I/21	CRC
24	Róna 1	I/31	CRC
25	GK Áron	I/32	CRC
26	GK Balázs	I/33	CRC
27	GK Erick	I/34	CRC
28	A119×KS61B) ×SMRIL	I/35	CRC
29	A119×KS60B) ×SMRIL	I/36	CRC
30	AIL-1× B119×Va-Cir	I/37	CRC
31	GK Csaba	I/201	CRC

3.1.2 Genomic DNA extraction

Total genomic DNA of the East African genotypes was extracted following Aqua Genomic ™ plant protocol according to MoBiTec GmbH 2012. Fourteen days old young fresh leaves of each genotype were harvested and weighed to 20 mg. The leaves were then cut into small pieces and placed in a 200 µl Aqua Genomic buffer solution in a pestle and tube homogenizer. The plant tissues were homogenized at room temperature. After homogenization, a tenth of the volume was added to 100% isopropanol to reduce foaming of the sample.

The samples were briefly vortexed and immediately poured into 1.5 ml microfuge tubes. Pelleting the debris involved centrifuging the samples at 10,000-20,000 xg for 4 minutes. The clear supernatant approximately 100 µl was transferred to fresh 1.5ml microfuge tubes followed by addition of 300 µl of hundred percent iso-propanol. The contents were mixed by vortexing for 30 seconds. Centrifuging at 10,000-20,000 xg for 4 minutes allowed the DNA to pellet. The samples were then flipped to discard the supernatant followed by filling the microfuge tube with 70% ethanol which was shot at the cap of the tubes from a squeeze bottle and then flipped two times.

Blotting was done on a paper towel several times to remove residual ethanol. The DNA pellets were then air-dried followed by addition of 50 µl TE buffer and pipetting up and down and vortexing vigorously to suspend the DNA. Centrifugation was again done for 2 minutes to ensure all the insoluble DNA pellets were dissolved. The clear DNA solution was transferred into a new tube then stored at 4 ºC in a refrigerator awaiting quality and concentration determination. 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 of DNA extraction, although it produced low quantities of genomic DNA, of relatively low purity as compared to the Omega™ kit.

3.1.3 Quality and concentration determination of genomic DNA

The genomic DNA was confirmed by running it on 1.0% agarose gel stained with ethidium bromide (1%) 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 µl after normalization to 10 ng/µl.

3.1.4 SSRs markers and genomic DNA amplification using PCR

Out of 41 SSR markers used by Billot et al. (2013), only five primer pairs that were highly polymorphic with a high allele number and at different chromosome numbers were selected

(Mwangi 2019). The forward primers were labelled with fluorescent dye (CY5) while the reverse primers were unlabeled (Tab. 5).

Table 5. Details of the SSR primers used in the study

Marker name	**Sequences**	**Repeat motifs**	**Size range (bp)**
xgap-206	5'ATTCATCATCCTCATCCTCGTAGAA 3' 3' CAAAAACCAACCCGACCCACTC 5'	(AC)13/(AG)20	106-146
mSbCIR286	5' GCTTCTATACTCCCCTCCAC 3' 3' TTTATGGTAGGATGCTCTGC 5'	(AC)9	112-134
mSbCIR248	5' GTTGGTCAGTGGTGGATAAA 3' 3' ACTCCCATGTGCTGAATCT 5'	(GT)7.5	89-101
mSbCIR262	5' GCACCAAAATCAGCGTCT 3' 3' CCATTTACCCGTGGATTAGT 5'	(CATG)3.25	208-220
mSbCIR238	5' AGAAGAAAAGGGGTAAGAGC 3' 3' CGAGAAACAATTACATGAACC 5'	(AC)26	79-107

PCR amplification reactions were done in 10 µl reaction 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 ℃ 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, 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.

3.1.5 Gel casting and selection of Taq DNA polymerase enzyme

Agarose gel was prepared by adding 1 g agarose into 100 ml of 0.5×TBE buffer in a conical flask and heating it (boiling) until dissolution of the agarose in a microwave for 1 minute followed by cooling by running cold water on the flask. Three µl of ethidium bromide solution (0.5 µg/mL) was added and the mixture put in an assembled gel electrophoresis casting tray with a well comb mounted on it. The mixture allowed to solidify for 20 minutes; the gel casting tray was put in gel electrophoresis tank.

Two µl of 1 kb molecular weight marker was loaded to the 1st well, while 5 µl PCR products from 5 samples prepared in two sets with each a different Taq DNA polymerase enzyme (Dream Taq™ or West Team Taq™) mixed with 2 µl loading dye was loaded into other wells on two different agarose gels in electrophoresis tanks. The samples were run at 110 V for 15 minutes, and thereafter gel images were taken under UV light (Fig. 5).

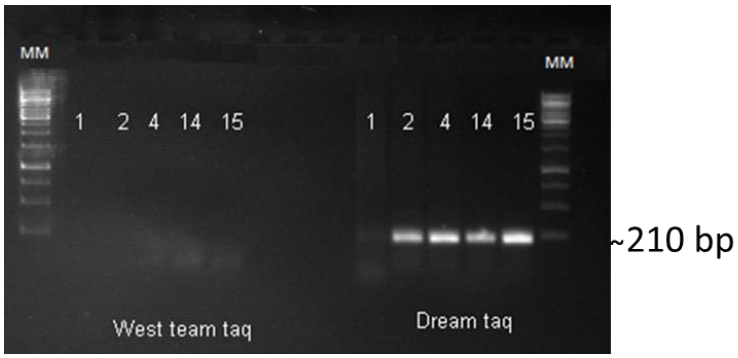

Figure 5. Tests of Dream Taq™ and West Team Taq™ polymerase with PCR amplicons from 5 samples on 1% ethidium bromide-stained agarose gel with primer mSbCIR 262 against MM: Thermo Fisher Scientific Gene Ruler 1 kb ladder.

3.1.6 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 in the 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 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.

3.1.7 Polyacrylamide Gel Electrophoresis (PAGE) with ALF Express II Fragment Analyzer™ machine

Preparation for polyacrylamide gel electrophoresis with Alf Express II Fragment Analyzer™ machine involved cleaning the thermoplate, glass plate, spacers and combs by washing properly using sterile water and blot drying them. The appropriate spacers with a thickness of 0.5 mm were placed at each side of the thermo plate as this determined the thickness of the gel. 90 µl of the bind silane was applied on the glass plate and spread appropriately and allowed first to dry. An appropriate comb was selected and inserted between the spacers and tested for fit and then removed. Clips were used to assemble and hold the glass plate on both sides. 6 % polyacrylamide gel (30 ml of 6% polyacrylamide gel consisting of 5X TBE buffer, urea, Acrylamide:Bis-Acrylamide (19:1), and water, was mixed with 30 µl of tetramethyldiamine (TEMED) and 30 µl of freshly prepared 25% ammonium-persulfate) was poured between the plates and then the appropriate comb was inserted.

The gel was polymerized by UV light for 15 minutes. After polymerization, the gel was transferred into ALFExpress II Fragment Analyzer™ machine, 1 L of 1×TBE buffer was added to each of the lower and upper tanks in the machine after which the comb was removed. The gel was first pre-run for 15 minutes until it reached an appropriate temperature, the wells were then rinsed with the buffer in the tanks in preparation for samples loading.

3.1.8 Preparation of samples for polyacrylamide gel electrophoresis

Two types of standards were used:

- external standard consisting of 70, 95, 150, 275 and 300 bp size fragments of 7.5µl from each and 262.5 µl sterile deionized water to a final volume of 300 µl serving as the molecular weight markers;
- internal standard comprising of 70 or 95 and 300 bp size fragments determined by the size of the sample;

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 (1×) 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.

3.1.9 Detection of the PCR product on ALF Express Fragment II Analyzer™ machine

The samples were run for 50 minutes at 850 V,75 mA, 65 W at constant temperature of 55 ℃. On completion of the 1st run, the wells were washed with the buffer and a repeat of the samples was loaded for the 2nd run and the same process was repeated for the 3rd run. The ALF Express II Fragment Analyzer™ machine contains a laser which excites the fluorescent labelled forward primers in the PCR product, this excitation results in the ALFwin fragment Analyzer 1.0 software creating peaks. The peaks of the external and internal standards were used to determine the correct allele sizes of the microsatellite loci.

3.1.10 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 were converted into binary codes and dendrogram constructed with SPSS version 23.

3.2 Experiment on the suitability of 2,4-D and TDZ for the induction of somatic embryogenesis of sorghum

3.2.1 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 the Cereal Research Non-profit Ltd., Szeged, Hungary.

3.2.2 Sterilization

The unopened stalk segments were cut in the laboratory at about 8 cm towards the end containing the floral meristem. The stalk segments that included the young inflorescence were disinfected 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 disinfected stalk segments were then placed upright in sterilized and capped glass bottles under a laminar air flow cabinet.

3.2.3 Assessment of the hormones with induction media

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 in 90×15 mm Petri dishes containing induction medium, half strength MS medium (Murashige and Skoog, 1962) supplemented with 2 mg/L 2,4-D, 2 mg/L TDZ, 5 mg/L 2,4-D and 5 mg/L TDZ labelled as T1, T2, T3 and T4, respectively (Tab. 6). The Petri dishes were incubated in the dark thermostat at 28 °C for 5 weeks and the number of the type of calli formed out of the 10 explants in each Petri dish and their colour was recorded each week.

Table 6. Induction, regeneration and rooting media used in the study

Callus induction medium annotation	**T1**	**T2**	**T3**	**T4**
Basic medium	2.2 g MS powder (MS0222)	2.2 g MS powder (MS0222)	2.2 g MS powder (MS0222)	2.2 g MS powder (MS0222)
2,4-D	2 mg	0 mg	5 mg	0 mg
TDZ	0 mg	2 mg	0 mg	5 mg
Sucrose	20 g	20 g	20 g	20 g
pH	5.8	5.8	5.8	5.8
Gelrite®	2.8 g	2.8 g	2.8 g	2.8 g
Regeneration medium	**R1**	**R2**	**R3 (190-2 Cu)***	
Basic medium	4.4 g MS powder (MS0222)	4.4 g MS powder (MS0222)	-	-
Zeatin	1.0 g	-	-	-
6-benzylaminopurine	1.0 g	1.0 g	-	-
Indole-3-acetic acid	-	1.0 g	-	-
$CuSO_4 \times 5H_20$	0.16 mg	0.16 mg	-	-
Sucrose	30 g	30 g	-	-
Gelrite®	2.8 g	2.8 g	-	-
pH	5.7	5.7	-	-
Rooting medium	190-2 Cu*			
*190-2Cu medium composition is shown in Table.1				

3.2.4 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.

3.2.5 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 on regeneration media:

- MS supplemented with cytokinin hormones zeatin and 6-benzylaminopurine (BAP) and $CuSO_4 \times 5H_2O$ denoted as R1;
- MS supplemented with auxin indole-3-acetic acid (IAA), BAP and $CuSO_4 \times 5H_2O$ [All the supplements were filter-sterilized into the medium after autoclaving, following the protocol described by Liu et al. (2015)] denoted as R2;
- 190-2 Cu medium (Tab. 1) denoted as R3 (Tab. 6);

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 Cu medium (Pauk et al. 2003). in glass and incubated under light for rooting induction. Data of the regenerants in the three-regeneration media were analyzed with R Commander X64 3.4.4 edition software.

3.3 Optimizing tissue culture media for sorghum somatic embryogenesis

3.3.1 Plant materials

Ten sorghum [*Sorghum bicolor* (L.) Moench] 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 of the Cereal Research Non-Profit Ltd., Szeged, Hungary.

3.3.2 Sterilization of plant explants

A set of 100 mature seeds from each genotype was disinfected separately by soaking them in 70% ethanol for 2 minutes and followed by agitation in 4% sodium hypochlorite and two drops of 'Tween 20' (in 100 ml) 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.

3.3.3 Comparison of the induction media

To test the efficacy of the six medium types (Tab. 7) in callus induction, twenty 1 mm pieces of mesocotyls from 1.5-day old germinating embryos were placed on each medium type contained in 90×15 mm plastic Petri dishes (Sarstedt, Newton, MA, USA) (Fig. 6). 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.

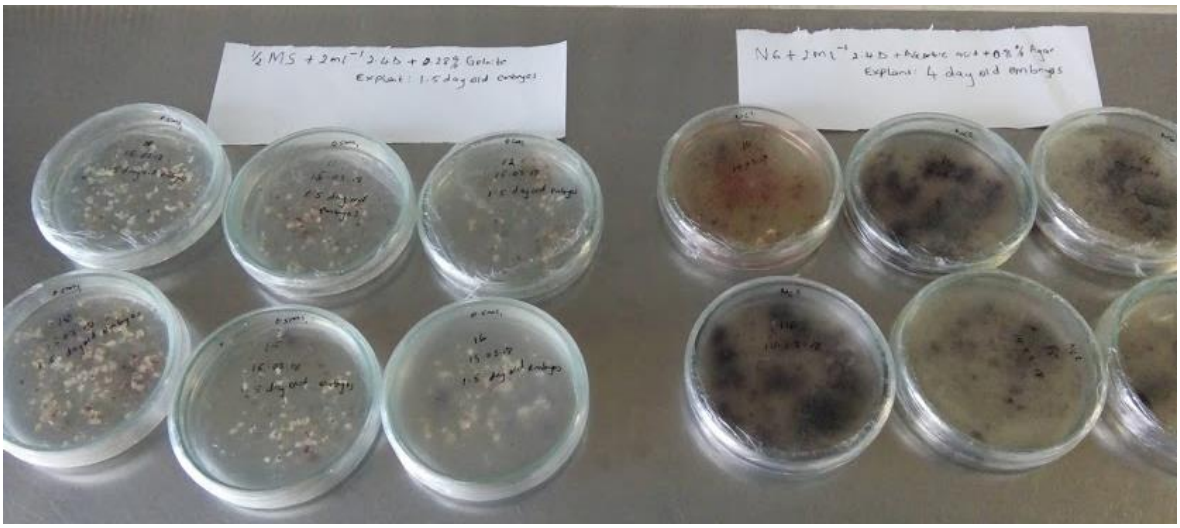

Figure 6. Sorghum callus induction on different medium types: left- 1 mm pieces of 1.5 day old germinating mesocotyls placed on half strength MS medium; right- 1mm pieces of 4 days old germinating mesocotyls on Chu-N6 medium.

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.

3.3.4 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 7. 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), 2 mg/l 2,4-D, 20 g/l sucrose, 2.8 g/l Gelrite, pH 5.7
B	½ 190-2 Cu (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, 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

3.4 Doubled haploid production using an improved anther culture protocol for sorghum [*Sorghum bicolor* (L.) Moench]

3.4.1 Plant material

Over a period of 24 months (summer of 2018, winter of 2018/2019, summer of 2019, winter of 2019/2020), twenty-eight Hungarian sorghum [*Sorghum bicolor* (L.) Moench] genotypes (Tab. 8), including F_1 registered- and experimental hybrids were planted in the field and in glasshouses in the summers and winters, respectively. The repeated plantings served as seasonal replications. All the sorghum genotypes were obtained from the sorghum breeding program of the Cereal Research Non-Profit 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.

Table 8. Sorghum registered F_1 hybrids and experimental ones used in the trial

	Geotype	Combinations
1	Experimental hybrids	A2KORAI×ZSV04/30, AREL×ZSV04/30, AREL×VSZ25KKD, ARET×VSZ25KKD, RE2A×(SRE1×SD100-F), ARET×ZSV04/30, SRE2A×SZETC73, AREL×SZE697/01, AREL×SZETC73, ARET×SZETC73, SRE2A× (A119×SZE 22-82), ARET×(SRE1×SD100-F), ARET×VSZ21KKD, A119×SZeTC73, (A119×KS61B) ×SMRIL 1/37, (A119×KS60B) ×SMRIL 1/38, (AIL-1×B119) ×VA-CIR 1/39
2	Registered hybrids	Alföldi 1, GK Zsófia, Albita, Farmsugro 180, Róna 1, GK Emese, GK Erzsébet, GK Áron, GK Balázs, GK Erik, GK Csaba

3.4.2 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 (Fig. 7), 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, 8 and 10 ˚C each for 7, 10 and 15 days in lighted phytotron chamber at 85% relative humidity prior to sterilization. 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 sterilized 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.

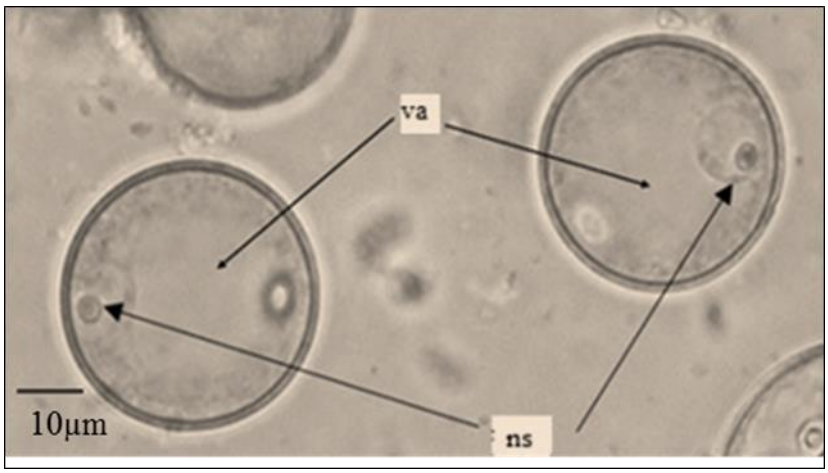

Figure 7. *Sorghum bicolor* (L.) Moench microspores at early vacuolated uni-nucleated stage: ns= nucleus, va = vacuole

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 in 90 mm × 15 mm plastic Petri dishes (Sarstedt, Newton, MA, USA) and placed at 28˚C in a 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.

3.4.3 Callus induction media

Three media (Tab. 9):

- 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 callus 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);
- 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 medium was M2, following results from the preceding summer's work.

3.4.4 Regeneration and hardening

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

3.4.5 Ploidy-level determination and chromosome doubling

On attaining the 4-leaf stage, the ploidy level of all regenerants was determined directly through flow cytometry. Pieces of fresh young leaves weighing 50 mg were harvested into Petri-dishes then transferred into 2 ml Eppendorf tubes followed by addition of 1ml Galbraith buffer (Galbraith et al. 1983). The leaves in the buffer were then broken down by shaking them in a 300 cycles per minute homogenizer (Tissuelyser II, Qiagen®). After a 15-minute wait for the foam to subside, the homogenate was filtered into a cleant 2 ml Eppendorf tube and a 10 µl RNase added to obtain an RNA free filtrate. This was followed by a 30-minute wait, after which 40 µl propidium iodide (1 mg/ml PI) dye was added. After another 30 minutes wait, measurements were taken using the Beckman Coulter Life Sciences CytoFLEX benchtop flow cytometer, which had been blanked with 1 ml deionized water. Like in the indirect method above, diploid counterparts of the regenerants were included in the tests as control.

A confirmatory test involving DNA extraction and PCR with selected SSR markers was also conducted for some of the regenerants by determining allele sizes following Polyacrylamide Gel Electrophoresis (PAGE) on ALFExpress II DNA fragment analyser machine for checking the homozygosity of the regenerants (Billot et al. 2013).

Colchicine treatment was applied for two plants of ‘Róna 1’ regenerants from the induction work of the winter of 2018/2019. The roots of the plants were cut back to about 10 mm from the crown before the plants were thoroughly washed with running tap water and dried for 1 hour. The plants were then dipped into previously prepared 0.05% colchicine solution in an upright position for 5 hours, in glasshouse at 24-26 ˚C. The 0.05% colchicine solution had been prepared by adding 10 ml tap water into a bottle containing 5 mg colchicine and 200 µl or 2% dimethylsulfoxide (DMSO). The plants were then placed under running water overnight and thereafter transplanted to sand and peat soil (1:1) mixture in plastic pots and covered with plastic bags, a space just enough for air circulation being left at the bottom and watered appropriately for a week before the glass cover was removed. 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. The fertility levels of the panicles were determined by the average number of filled grains of three randomly selected panicles from each of the originalthree 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.

Table 9. Components of the three callus induction media in the sorghum anther culture experiment in the summer of 2018 and winter of 2018/2019

Components	**Media (mg/L)**		
	MS-t-z-2	N_6	W14mf
KNO_3	1,900	2,830	2,000
KH_2PO_4	170	400	-
NH_4NO_4	1,650	-	-
$NH_4H_2PO_4$	-	-	380
$(NH_4)_2SO_4$	-	463	-
$CaCl_2.2H_2O$	-	166	140
$CaCl_2$	332	-	
$MgSO_4.7H_2O$	-	185	200
(or $MgSO_4$)	180	-	-
K_2SO_4	-	-	700
Na_2.EDTA	-	37.3	-
$FeNa_2$-EDTA	36.7		36.7
$FeSO_4$ x$7H_2O$	-	27.8	-
$MnSO_4$ x $4H_2O$	-	0.8	8
(or $MnSO_4$ x H_2O)	16.9		-
$ZnSO_4$ x $7H_2O$	8.6	0.3	3
H_3BO_3	6.2	0.32	3
KI	0.83	0.16	0.5
$CuSO_4$ x $5H_2O$	0.025	-	0.025
$CoCl_2$ x $6H_2O$	0.025	-	0.025
Na_2MoO_4 x $2H_2O$	0.25	-	0.005
Glycine	2	2	
Thiamine.HCl	0.1	1	1
Pyridoxine.HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Myoinositol	100	-	-
Casein Hydrolysate	-	-	-
Biotin	-	-	-
2,4-D	2	2	2
NAA	-	-	
Kinetin	0.3	0.5	0.5
Zeatin	2.2	-	-
Maltose (Sigma)	20,000	-	80,000
Sucrose	-	30,000	-
Agar	5,000	-	-
Ficoll®400	-	-	100,000
Gelrite	-	2,800	-
pH	5.8	5.8	5.8

4. RESULTS

4.1 Assessing the genetic diversity of some selected Hungarian and East African genotypes

In this research, five sorghum SSR primer pairs, (XGAP 206, mSbCIR 286, mSbCIR 248, mSbCIR 262 and mSbCIR 238) produced polymorphic amplified products for 31 Sorghum genotypes. The PCR products were tested on 1.5% ethidium bromide stained (Fig. 8) agarose gel. This was done to check whether the PCR products were amplified or not.

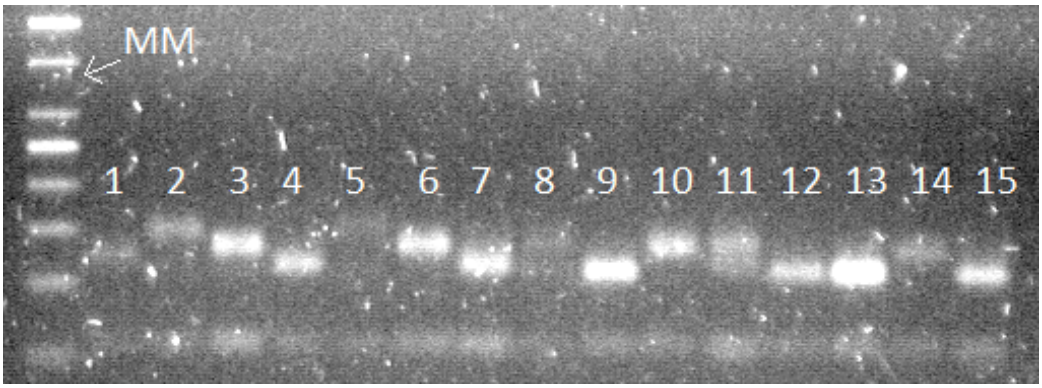

Figure 8. Amplification pattern of 15 Sorghum genotypes obtained by SSR marker Xgap-206 on 1.5% ethidium bromide-stained agarose gel. Numbers 1-15 represent genotype serial numbers as shown in Tab. 4.

MM: Thermo Fisher Scientific GeneRuler 100 bp Ladder Plus.

Polyacrylamide gel electrophoresis was used to detect the exact allele sizes using ALF Express II Fragment Analyzer™ machine. An example of the size determination with the ALF win software analysis 1.0 is shown in Fig. 9.

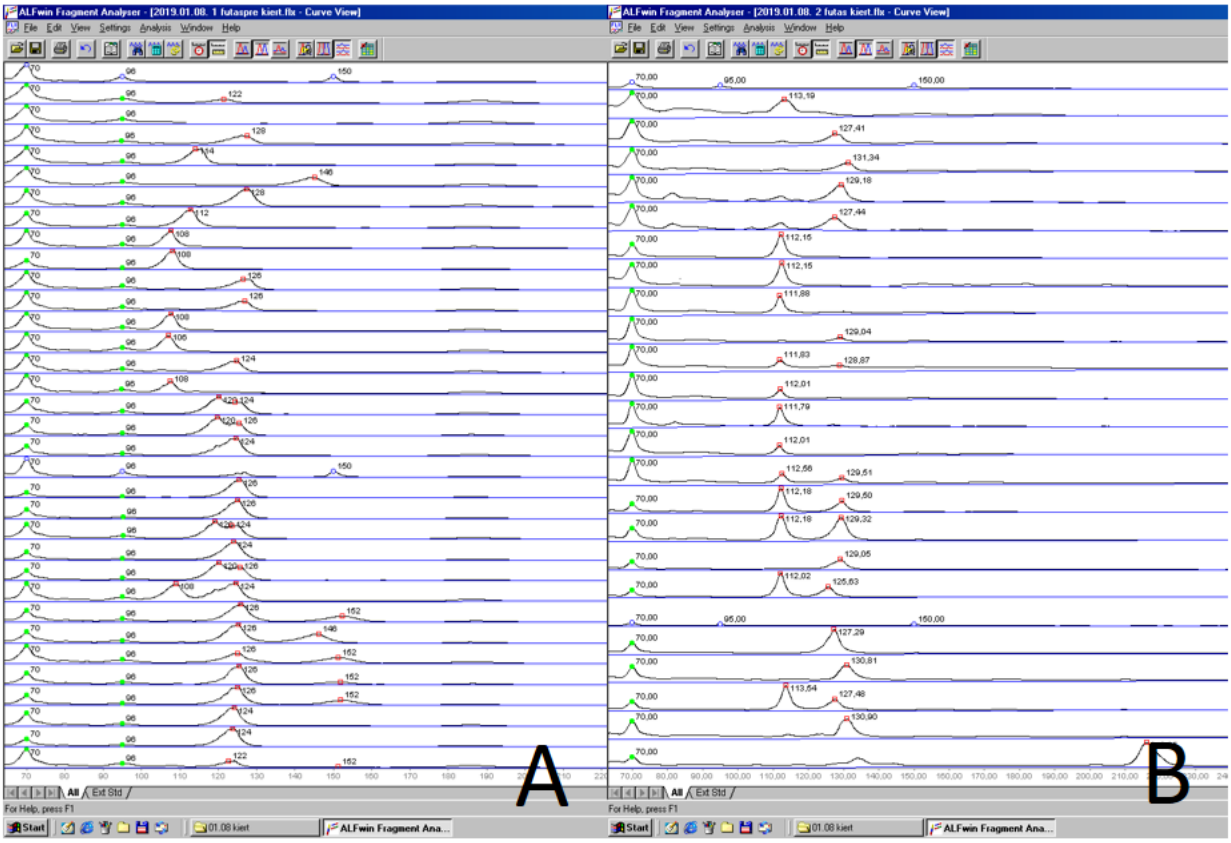

Figure 9. Peaks of the polyacrylamide gel electrophoresis analysis with Alfwin Fragment Analyzer 1.0 software with primers (a) Xgap-206 and (b) mSbCIR286.

The PCR products were further observed on 2% NuSieve ethidium bromide stained gel (Fig. 10).

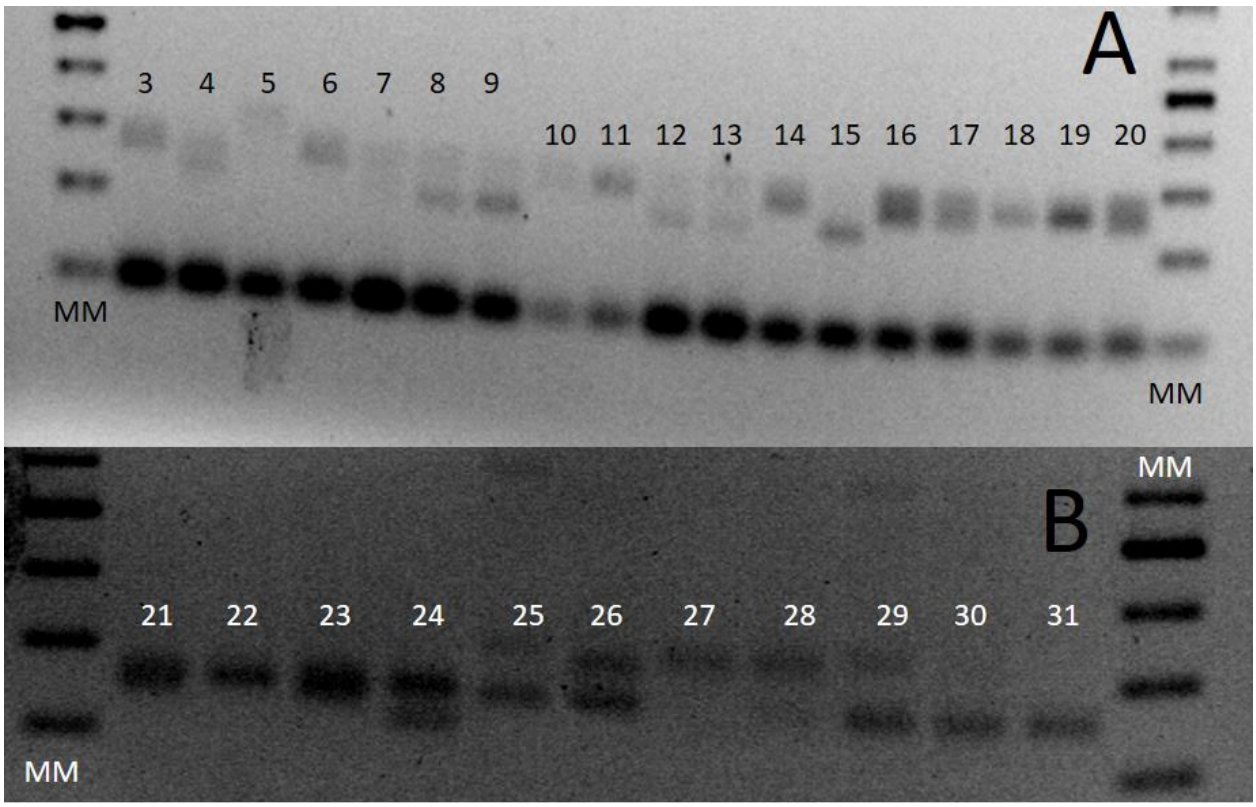

Figure 10. (A) and (B): Ethidium bromide (1%)stained 2% NuSieve agarose gel photo of the PCR products generated with primer Xgap-206. The numbers 3-31 are the genotype serial numbers (with the exception of 1 and 2) as shown in Table. 3.

MM: Thermo Fisher Scientific GeneRuler 50 bp DNA Ladder,

The allele sizes determined by ALF Express machine are collected in Tab. 10. 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.

Table 10. Correct allele sizes (bp) of 31 analyzed sorghum genotypes at 5 SSR loci - adapted from Mwangi (2019)

SSR Loci		Alleles (bp)				
		Xgap 206	**mSbCIR 286**	**mSbCIR 248**	**mSbCIR 262**	**mSbCIR 238**
1	ICSR93034	120:120	112:112	102:102	216:216	89:89
2	Framida	126:126	130:130	102:102	216:216	89:89
3	Seredo	126:126	112:112	102:102	216:216	89:89
4	Hakika	114:114	130:130	102:102	216:216	89:89
5	EC-Teso	146:146	130:130	94:94	216:216	81:81
6	Mahube	126:126	130:130	102:102	216:216	81:81
7	KARI Mtama 1	114:114	126:126	102:102	216:216	75:75
8	Abaleshya	108:108	112:112	102:102	216:216	75:75
9	IESV91054LT	108:108	112:112	102:102	216:216	75:75
10	F6YQ212	126:126	112:112	102:102	216:216	89:89
11	Gadam el Hamam	126:126	130:130	94:102	216:216	89:89
12	Cytanombe	108:108	112:130	102:102	216:216	75:75
13	SV900I5LT	108:108	112:112	102:102	216:216	75:75
14	IESV91069LT	126:126	112:112	102:102	216:216	81:81
15	Ndamoga	108:108	112:112	94:102	216:216	75:75
16	Alföldi 1	120:126	112:130	102:102	216:216	75:89
17	GK Emese	120:126	112:130	94:94	216:216	75:89
18	GK Zsófia	126:126	112:130	94:94	216:216	75:75
19	SREZA× (A119 × SZc	126:126	130:130	102:102	216:216	75:83
20	ARET×VSZ21KKD	126:126	112:126	102:102	216:216	75:89
21	AREL×VSZ21KKD	120:126	126:126	94:94	216:216	75:89
22	A119 × SZeTC73	126:126	130:130	94:102	216:216	75:83
23	Farmusugro 180	120:126	112:126	94:102	216:216	75:89
24	Róna 1	108:126	130:130	102:102	216:216	75:83
25	GK Áron	126:152	112:126	102:102	216:216	75:75
26	GK Balázs	126:146	126:126	102:102	216:220	75:75
27	GK Erick	126:152	126:126	102:102	216:220	81:81
28	(A119×KS61B)	126:152	112:126	102:102	216:216	75:75
29	(A119×KS60B)	126:152	112:126	94:102	216:216	75:75
30	AIL-1× B119×Va-Cir	126:126	112:130	102:102	216:216	75:75
31	GK Csaba	126:126	112:130	102:102	216:216	75:75

A dendrogram **(**Fig.11) was constructed based on the SSR data.

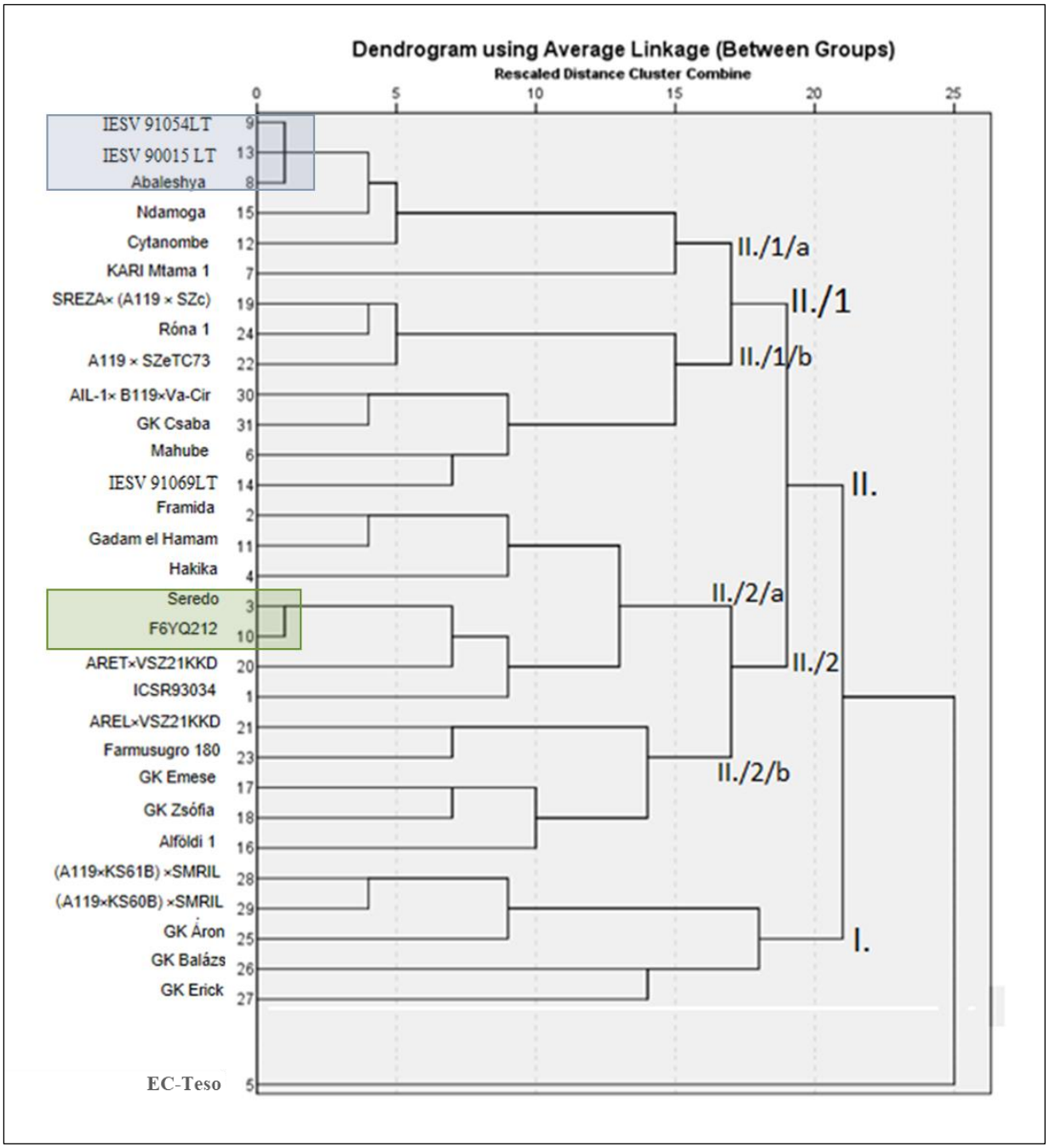

Figure 11. Dendrogram based on microsatellite analysis results of 31 Sorghum genotypes (15 East African and 16 Hungarian genotypes).

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. Genetically similar genotypes were clustered together. The cluster analysis resolved the 31 sorghum genotypes into two major clusters at 16% similarity threshold (Fig. 11**)** excluding genotype 5 which was dissimilar to the rest of the germplasm**.** The number of genotypes in each

of the two clusters is shown in Tab. 11. The cluster comprised of genotypes 25,26, 27, 28, 29 and the rest of the 30 germplasms in cluster I and II respectively. Cluster I comprised exclusively of genotypes sourced from the Hungarian breeding program. Cluster II on the other hand comprised of a mix of Hungarian and East African germplasms at 60, 64 and 72% similarity thresholds. They included 16 (East African), 17 and 18 (Hungarian) at 60% similarity threshold, germplasms 6, 14 (East African) and germplasm 30, 31 (Hungarian) at 64% and the third comprising germplasms 3, 10 (East African) and germplasm 20 (Hungarian) at 72% similarity threshold.

Cluster II formed 4 sub-clusters at 32% similarity threshold with 2 sub-clusters comprising 6 germplasms each while the other 2 sub-clusters comprised of 7 germplasms. Germplasms 8, 9, 13 in one cluster and 3, 10 are highlighted (Fig.11) as it was not possible to distinguish them with the limited number of markers used. It was observed that for greater genetic distance determination, more markers than those used in the present study would be necessary.

Table 11. Clustering pattern of 31 Sorghum genotypes based on molecular marker analysis

Cluster	**Number of genotypes**	**Genotypes**
0	5	EC-Teso
I	27, 26, 25, 29 and 28	GK Erick, GK Balázs, GK Áron, A119×KS60B) ×SMRIL and A119×KS61B) ×SMRIL
II, II/1a	16, 18, 17, 23 and 21	Alföldi 1, GK Zsófia, GK Emese, Farmusugro 180 and AREL×VSZ21KKD.
II, II/1b	1, 20, 10, 3, 4, 11, and 2	ICSR93034, ARET×VSZ21KKD, F6YQ212, Seredo, Hakika, Gadam el Hamam and Framida.
II, II/2a	19, 24, 22, 30, 31, 6 and 14	SREZA× (A119 × SZc 2282), Róna 1, A119 × SZeTC73, AIL-1× B119×Va-Cir, GK Csaba, Mahube and IESV91069LT
II, II/2b	7, 12, 15, 8, 13 and 9	Kari Mtama 1, Cytanombe, Ndamoga, Abaleshya, IESV90015LT and IESV91054LT

4.2 Suitability of 2,4-D and TDZ for the induction of somatic embryogenesis of sorghum

In this study, modifications of half strength MS medium with 2 mg/L 2,4-D (T1), 2 mg/L TDZ (T2), mg/L 2,4-D (T3) and 5 mg/L TDZ (T4) respectively were applied in the embryogenic callus generation of 10 selected Hungarian sorghum genotypes from their young inflorescence explants with the aims of assessing the effect of modifications on half strength MS media with various quantities of hormones auxin 2,4-D and cytokinin TDZ, the effectiveness of young inflorescence as an explant and explant and media interaction for an embryogenic callus generation.

4.2.1 Differences of the callus types recovered

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) (Fig.12). EC of 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 (Fig. 13). The results therefore major on the EC% means as influenced by the four media, genotype as well as the period under incubation.

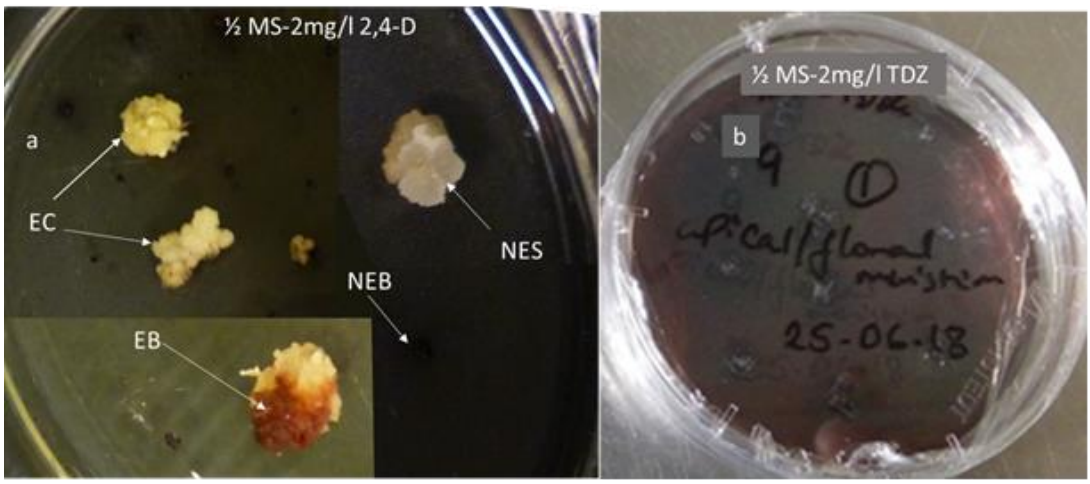

Figure 12. Different callus types induced from young inflorescence of sorghum: (a) Embryogenic Calli (EC), Embryogenic Brown callus (EB), Non-Embryogenic Soft callus (NES) and Non-Embryogenic Brown callus (NEB) (b) Callus induction on half-strength MS medium with 2 mg/LTDZ.

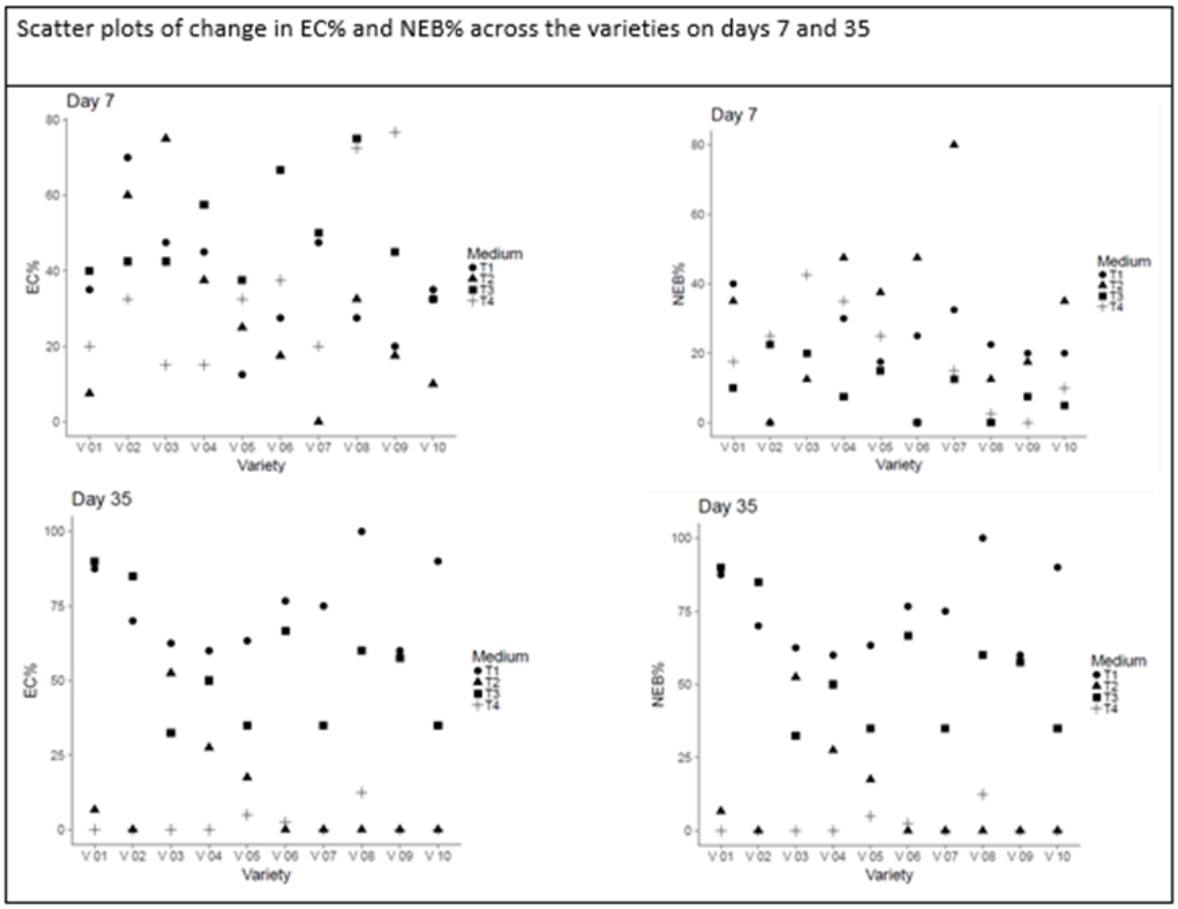

Figure 13. Scatter plot showing the change in EC% and NEB% means in the 10 varieties with the four treatments between day 7 and day 35.

4.2.2 Genotype vs medium type influence in the callus type recovered.

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 (Fig. 13, Tab. 12) to embryogenic character, thereby making the EC% means at day 35 to be higher than at day 7.

Table 12. Means of Embryogenic Cream Calli (EC) percentages as observed from Day 7 through to Day 35 in the 10 selected sorghum varieties

Genotype No.	Name of Genotype	EC% (means of four treatments/ medium types T1, T2, T3, T4)				
		Day 7	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 were higher than that of the other callus types (Fig. 13). The EC% at day 35 for T1 were higher than and significantly different from those of treatment T2, T3 and T4 (Fig. 14). EC% mean of treatment T3 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. This

can be seen by the negative gradient of the graphs of T2 and T4 (Fig. 15). The gradient in T4 was sharper than that of T4, which means that the EC turned brown very fast. The EC% means for T2 and T4 were not significantly different (Fig. 15) at day 28 and day 35. They were however different at day 7, 14 and 21 with those of T2 being higher than those of T4.

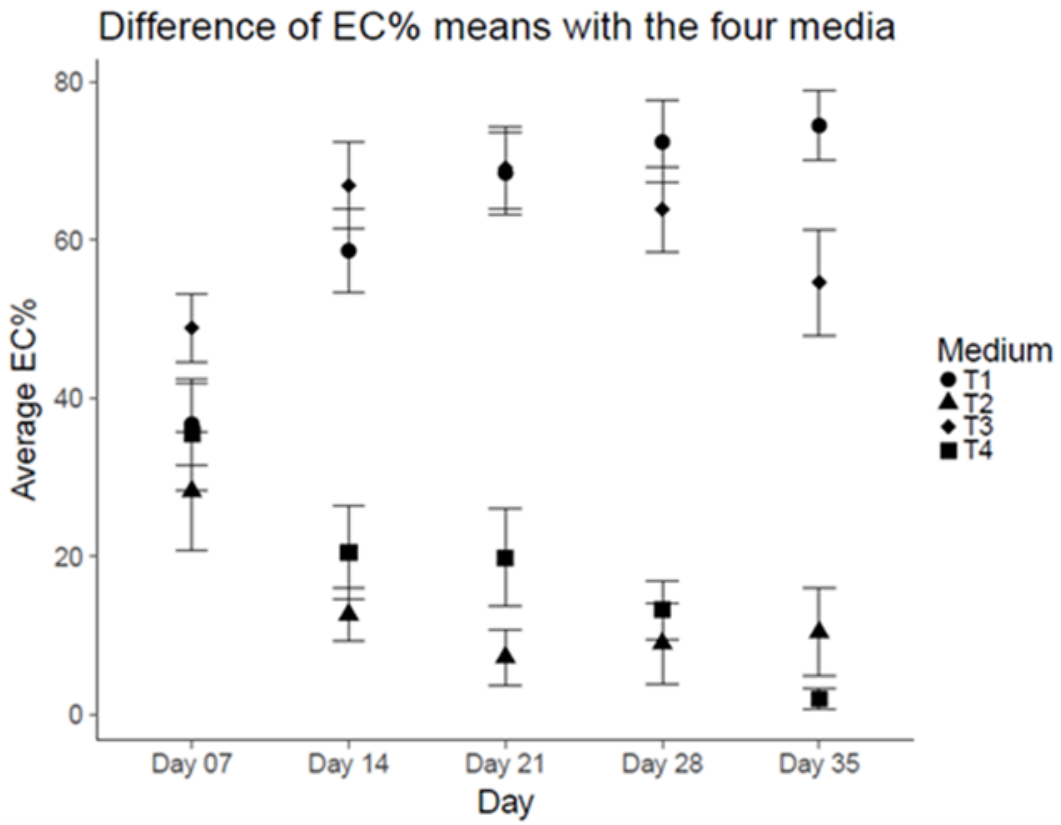

Figure 14. Error bars showing differences in EC% means for media T1, T2, T3 and T4

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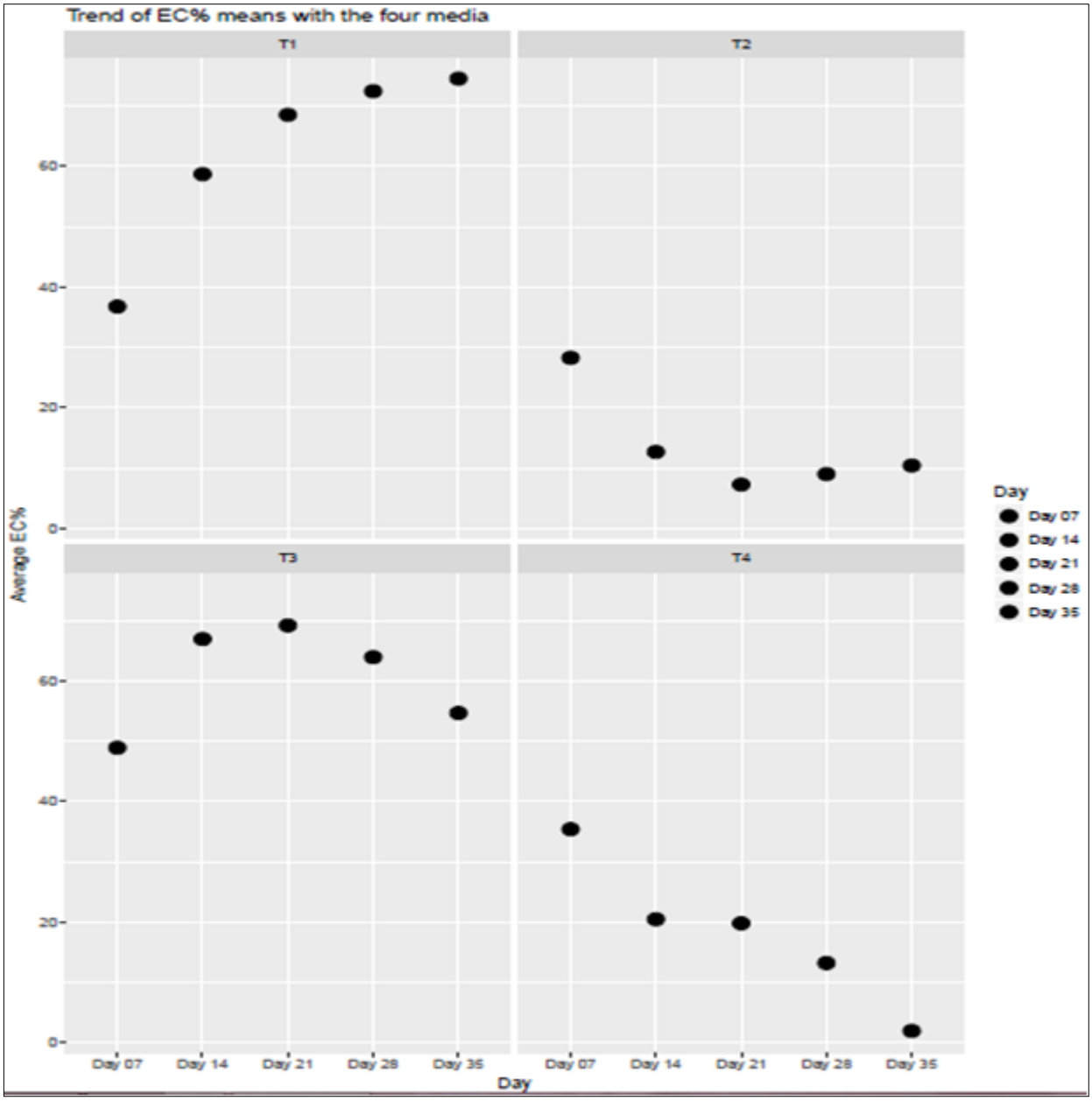

Figure 15. Graphs of average EC% with media T1, T2, T3 and T4 as observed in the five weeks period.

4.2.3 Regeneration of the quality calli recovered

Genotypes ‘Róna 1’, ‘GK Zsófia’ and ‘GK Emese’ regenerated, while the rest of the 10 genotypes failed to regenerate. Regeneration frequency was the highest with genotype ‘Róna 1’ in 190-2 Cu medium at 40%, followed by ‘GK Zsófia’ in the same medium at 33%. ‘GK Zsófia’ had the highest average number of shoots per explant (5) in medium R2 followed by ‘Róna 1’ with 4 shoots per explant in medium R3. The latter also produced the highest number of roots per explant with across the genotypes (Tab. 13) and therefore selected as the preferred rooting medium among the three (Fig. 16).

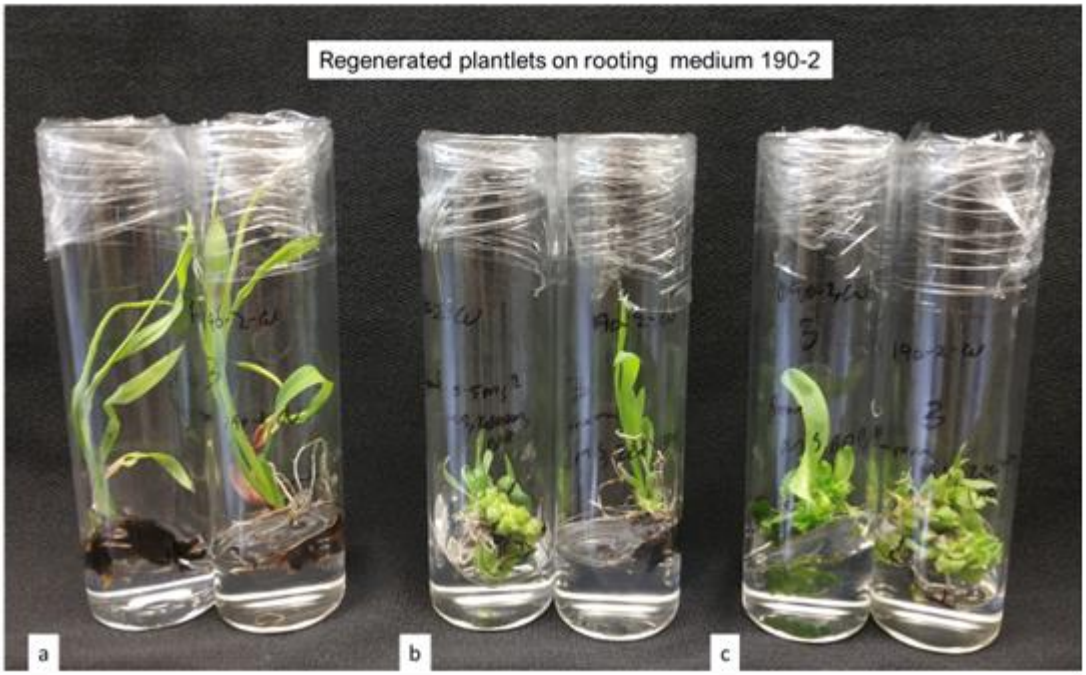

Figure 16. Regenerated genotype ‘GK Zsófia’ on rooting medium 190-2Cu: **a** plantlets regenerated on medium 190-2Cu; **b** plantlets regenerated on medium MS medium supplemented with auxin indole-3-acetic acid (IAA), BAP and $CuSO_4 \times 5H_2O$; **c** plantlets regenerated on medium MS supplemented with cytokinin hormones zeatin and 6-benzylaminopurine (BAP) and $CuSO_4 \times 5H_2O$.

Table 13. Regeneration frequency, roots and shoots per explant with 3 regeneration media across 3 genotypes

Genotype	Medium	Regeneration frequency (%)	Shoots per explant	Roots per explant
GK Emese	R1	13.3	1	1
	R2	6.7	1	1
	R3	0	0	0
GK Zsófia	R1	6.7	3	3
	R2	26.7	5	5
	R3	33.3	2	3
Róna 1	R1	13.3	1	1
	R2	20.0	1	3
	R3	40.0	4	7

4.3 Optimizing tissue culture media for sorghum somatic embryogenesis

This study aimed at assessing the suitability of various induction media to produce embryogenic callus (yellow and friable) with high induction rates and reduced phenolic exudation. The six culture medium modifications: 3 based on Murashige and Skoog (MS) medium and one each based on Chu N6, Gamborg B5 and 190-2 media respectively were applied in the culture of mature embryos from 10 sorghum genotypes.

The six media showed significantly different ($P<0.05$) average yellow callus percentages. Medium type E (Tab. 14) 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 (Tab. 14). The control medium type F had a lower average yellow callus percentage than medium type E, but these two were not significantly different.

All the MS-based medium types had higher friable callus induction percentages which were significantly different from the non-MS-based types B, C and D (Tab. 14). 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. Medium E had a significantly lower average brown callus percentage at 40.3% than medium C which exhibited the highest average (Tab. 14).

Occurrence of brown callus was seen to be influenced by the genotype, with candidate 'AIL-1× B119×Va-Cir' having the highest at 80.2%. Hybrid 'GK Áron' had the highest average yellow callus frequency at 53.7% and the least average brown callus at 40.8%. On the contrary, friability of the callus (Tab. 14) was not influenced by genotypes ($P>0.05$). Medium E generally had the highest average yellow callus ratio across the genotypes (Fig. 17) followed by the control medium type F. All the MS-based medium types had generally the highest average friable callus percentage across the 10 genotypes, with medium type E having a higher quantity callus in 6 of the 10 genotypes (Fig. 17), compared to the control which had a higher average at only one genotype. Medium type E tied with control medium F for the highest average friable calli percentage score for variety 'GK Emese'.

Table 14. Comparison of the average induction rate of yellow, friable and brown calli across the six medium types and 10 genotypes. The different letters mark the significantly different values

Medium	**Avg. Yellow calli %**	**Avg. Friable calli %**	**Avg. Brown calli %**
A	27.6 "a"	22.1 "b"	59.8 "ab"
B	22.6 "a"	5.6 "a"	60.5 "ab"
C	19.3 "a"	1.0 "a"	71.5 "b"
D	31.9 "a"	0.7 "a"	59.8 "ab"
E	59.7 "b"	25.1 "b"	40.3 "a"
F	40.0 "ab"	19.0 "b"	58.3 "ab"
P value	0.000104 ***	0.0000000697 ***	0.0217 *
Genotype			
V01	42.4"ab"	20.2"a"	50.8"ab"
V02	51.7 "b"	18.2"a"	41.4"a"
V03	41.5"ab"	17.7"a"	46.7"a"
V05	32.3"ab"	11.1"a"	57.9"ab"
V09	30.8"ab"	15.7"a"	61.2"ab"
V10	53.7"b"	20.6"a"	40.8"a"
V12	23.1"ab"	3.2"a"	68.1"ab"
V14	21.2"ab"	6.2"a"	69.7"ab"
V15	12.5"a"	2.6"a"	80.0"b"
V16	26.0"ab"	6.9"a"	67.2"ab"

***- significant at 0.001 *- significant at 0.05

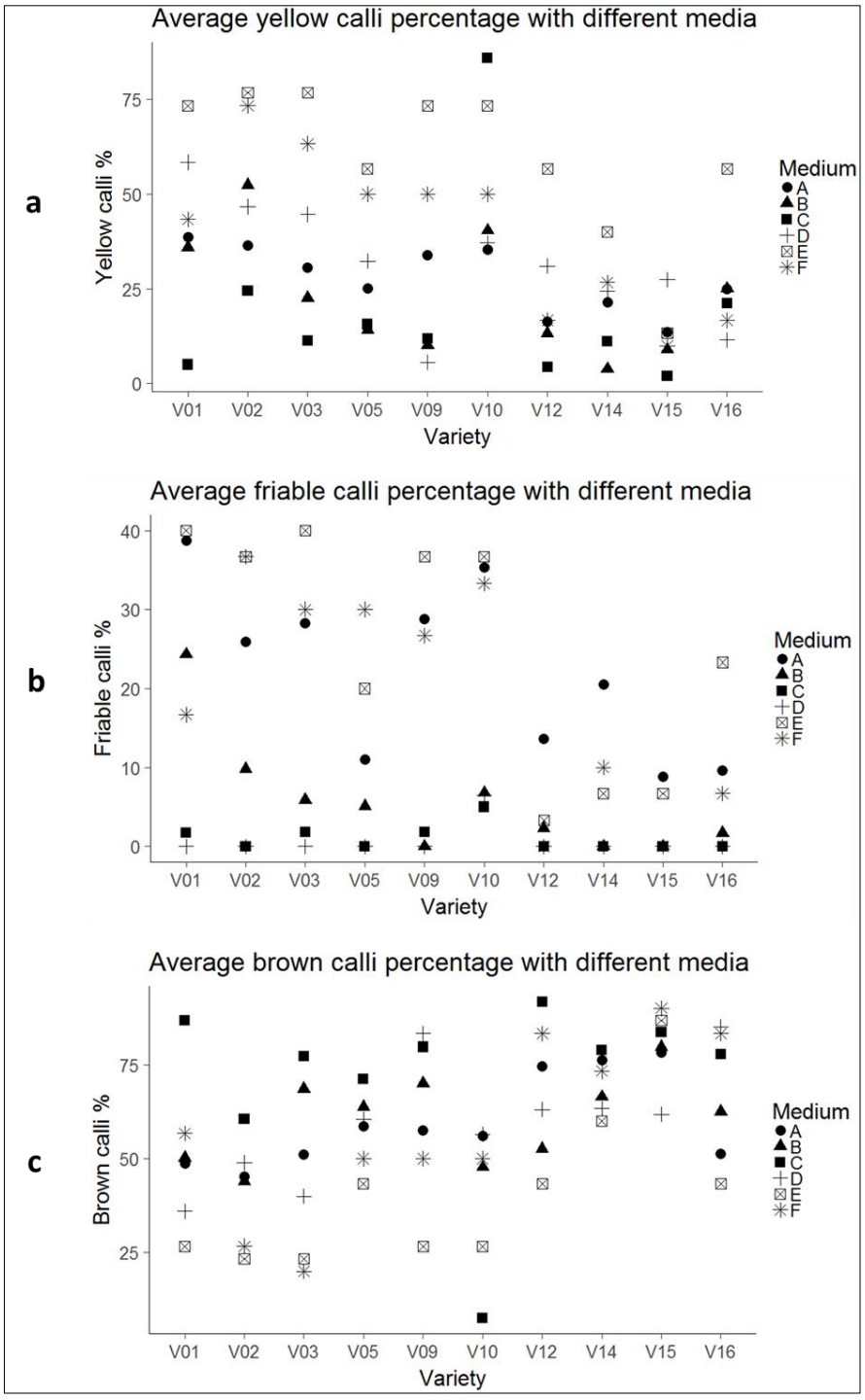

Figure 17. Scatter plots of callus induction percentages **a)** Average percentages of yellow **(b)** friable and (**c**) brown calli with the six medium types across the 10 genotypes.

4.4 Doubled haploid production using an improved anther culture protocol for sorghum [*Sorghum bicolor* (L.) Moench]

This study aimed at sorghum doubled haploids production thereby contributing to the development of an improved protocol using 28 hybrid genotypes, both F_1 registered- and experimental hybrids involved on different callus induction media, testing of various pre-treatment conditions, regeneration on 190-2 Cu medium, ploidy determination and chromosome doubling.

4.4.1 Recovery of embryogenic calli

Only four embryogenic calli were obtained from genotype 'GK Zsófia' on induction of the 36,000 anthers from the summer of 2018. The calli (Fig. 18) 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 190-2 Cu medium under light condition at 25±1 ˚C for 20-30 days and formed green plantlets.

Figure 18. Embryogenic calli forming from the anther culture of sorghum ('GK Zsófia') *in vitro*.

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. Similarly, to the results of the anthers incubated in the summer of the same year, the anthers that did not form calli discolored and died on incubation for 15 days. Only one of the 5 embryogenic calli from the induction work of the winter of the first year regenerated and proliferated on 190-2 Cu 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 (Fig. 19, Tab. 15). Out of these, 3 calli from 3 different genotypes (‘ARET×VSZ25KKD’; ‘AREL× SZE697/01’ and ‘AREL×ZSV04/30’) regenerated on 190-2 Cu 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 190-2 Cu regeneration medium on which they developed as individual plantlets.

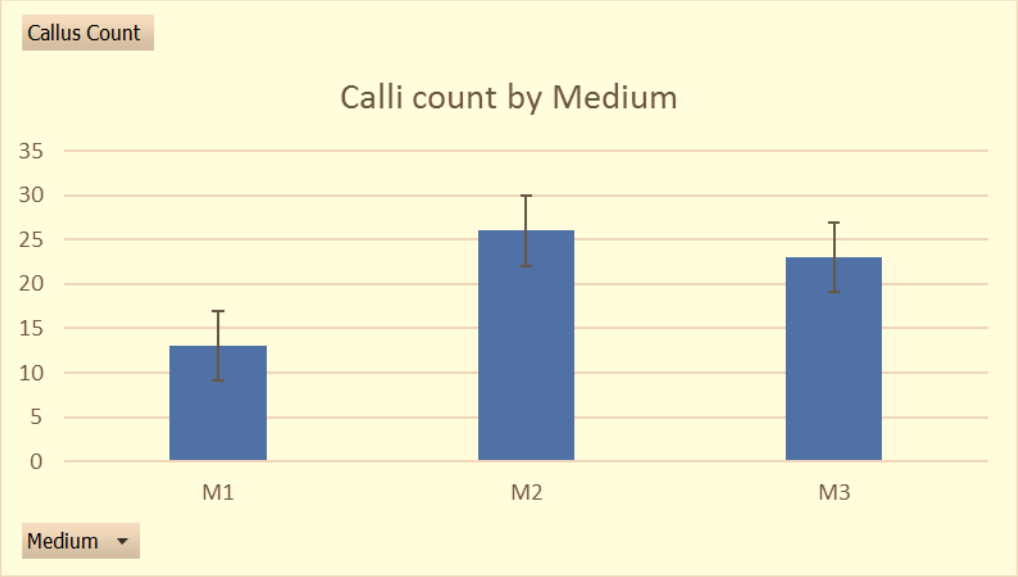

Figure 19. Differences in callus induction counts: the cumulative callus counts across the 3 different types of induction media in the summer of 2019 (sample size of 100 anthers per treatment in 3 medium types across the 9 responsive genotypes).

Table 15. Number of calli obtained on three anther culture media from sorghum F_1 registered (R)- and experimental (E) hybrids in the summer and winter of 2018 and summer of 2019

			Number of calli recovered by the summer of 2018(A), winter of 2018(B), summer of 2019(C) on different medium types								
S. No	**Genotype Name**	**Category of hybrid**	**M1**			**M2**			**M3**		
			A	B	C	A	B	C	A	B	C
1	Alföldi 1 st.	R	0	0	0	0	0	0	0	0	0
2	ARET×ZSV04/30	E	0	0	2	0	0	2	0	0	3
3	GK Zsófia	R	4	0	2	0	0	0	0	0	0
4	A2KORAI×ZSV04/30	E	0	0	0	0	0	6	0	0	1
5	AREL×ZSV04/30	E	0	0	0	0	0	0	0	0	1
6	AREL×VSZ25KKD	E	0	0	0	0	0	0	0	0	0
7	ARET×VSZ25KKD	E	0	0	1	0	0	9	0	0	6
8	SRE2A×(SRE1×SD100-F)	E	0	0	5	0	0	2	0	0	5
9	ARET×(SRE1×SD100-F)	E	0	0	0	0	0	0	0	0	0
10	SRE2A×SZETC73	E	0	0	2	0	0	0	0	0	1
11	AREL×SZE697/01	E	0	0	1	0	0	2	0	0	0
12	AREL×SZETC73	E	0	0	0	0	0	0	0	0	0
13	ARET×SZETC73	E	0	0	0	0	0	0	0	0	0
14	Albita	R	0	0	0	0	0	0	0	0	0
15	Farmsugro 180	R	0	0	0	0	0	0	0	0	0
16	Róna 1	R	0	5	1	0	0	5	0	0	0
17	GK Emese	R	0	0	0	0	0	0	0	0	0
18	SRE2A×(A119×SZE 22-82)	E	0	0	0	0	0	0	0	0	0
19	ARET×VSZ21KKD	E	0	0	0	0	0	0	0	0	0
20	GK Erzsébet	R	0	0	0	0	0	0	0	0	0
21	A119×SZeTC73	E	0	0	0	0	0	0	0	0	0

22	GK Áron	R	0	0	0	0	0	0	0	0	0
23	GK Balázs	R	0	0	0	0	0	0	0	0	0
24	GK Erik	R	0	0	0	0	0	0	0	0	0
25	(A119×KS61B)×SMRIL	E	0	0	0	0	0	0	0	0	0
26	(A119×KS60B)×SMRIL	E	0	0	0	0	0	0	0	0	0
27	(AIL-1×B119)×VA-CIR	E	0	0	0	0	0	0	0	0	0
28	GK Csaba	R	0	0	0	0	0	0	0	0	0

M1- Medium W14mf

M2- Medium W14mf supplemented with 1.0 g/l l-proline, 1.0 g/l l-asparagine and 1.0 g/l KH_2PO_4

M3- Medium W14mf supplemented with 1.0 g/l l-proline, 1.0 g/l l-asparagine but without KH_2PO_4

4.4.2 Pre-treatment for callus induction

As a result of the low number of calli obtained from the induction work of the summer of 2018 (4 from 'GK Zsófia' hybrid), all materials having been pre-treated at 4 ˚C for 10 days, the pre-treatment was applied in the season that followed. It resulted in only 5 embryogenic calli on W14mf medium as earlier explained in the induction work of the winter of 2018/2019, when anthers were pre-treated by holding them at 10˚C for 15 days in a lighted phytotron chamber at 85% relative humidity prior to sterilization (Tab. 16). Subsequently, this pre-treatment temperature and duration was used during the summer of 2019. In the winter of 2019/2020 however, having not obtained any major differences in the callus yield as a result of pre-treatment in the previous experiments, the anthers were induced directly upon collection from the plants.

The use of 0.3 M mannitol to effect starvation pre-treatment in the three media used in the summer of 2018 did not result in any callus induction. Similarly, no callus was induced on media N_6 and Mst-z-2, and therefore they were dropped in subsequent seasons i.e., winter of 2018/2019 and both summer and winter of 2019/2020, in favour of W14mf or modified versions of W14mf (M1, M2 and M3) that gave callus yields. In the summer of 2018, W14mf resulted in the induction of 4 embryogenic calli that were obtained from genotype 'GK Zsófia' whose panicles were obtained from field grown plants. On medium M2, 5 embryogenic calli were induced from the anthers of 'Róna 1' in the winter of the same year, panicles being obtained from glasshouse grown plants. Noteworthy, in this season, callus induction from 'GK Zsófia' was not reproduced.

Table 16. Evaluation of different pre-treatment temperatures in different durations for sorghum anther culture induction for registered hybrid ‘Róna 1’with a sample size of 100 anthers in 4 replications per treatment

Temperature (˚C)	7 days	10 days	15 days
4	0 calli	0 calli	0 calli
8	0 calli	0 calli	0 calli
10	0 calli	0 calli	5 calli

4.4.3 Effect of medium modification on callus induction

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. Medium W14mf supplemented with 1.0 g/l L-proline, 1.0 g/l L-asparagine and 1.0 g/l KH_2PO_4 (M2) led to the highest induction (26 embryogenic calli) although a Kruskal-Wallis test conducted for the induced calli among media M1, M2 and M3 showed no significant differences (Chi square = 1.53, df = 2, P = 0.46). M1 and M3 led to the induction of calli in 6 of the 9 responsive genotypes while M2 had induction on only 5 of the 9 responsive genotypes but had the highest number of calli (9) for one individual genotype (F_2 experimental hybrid ‘ARET×VSZ25KKD’). Following this outcome, a reproducibility test in the winter of 2019/2020 was done with M2 as the induction medium for only the 9 responsive genotypes. While all the 9 genotypes formed calli during this reproducibility test, only ‘AREL×ZSV04/30’ formed a green plantlet after its callus was transferred to the regeneration medium less than 7 days after induction, unlike in the previous season where the same genotypes yielded an albino plantlet.

4.4.4 Ploidy level determination

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. 17; Fig. 20). 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 (Fig. 21).

Table 17. 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		Xgap-206	
Genotype ‘Róna 1’	108	126	75	83
Genotype ‘Róna 1’	126	126	83	83
Genotype ‘Róna 1’	126	126	83	83
Genotype ‘Róna 1’	126	126	83	83

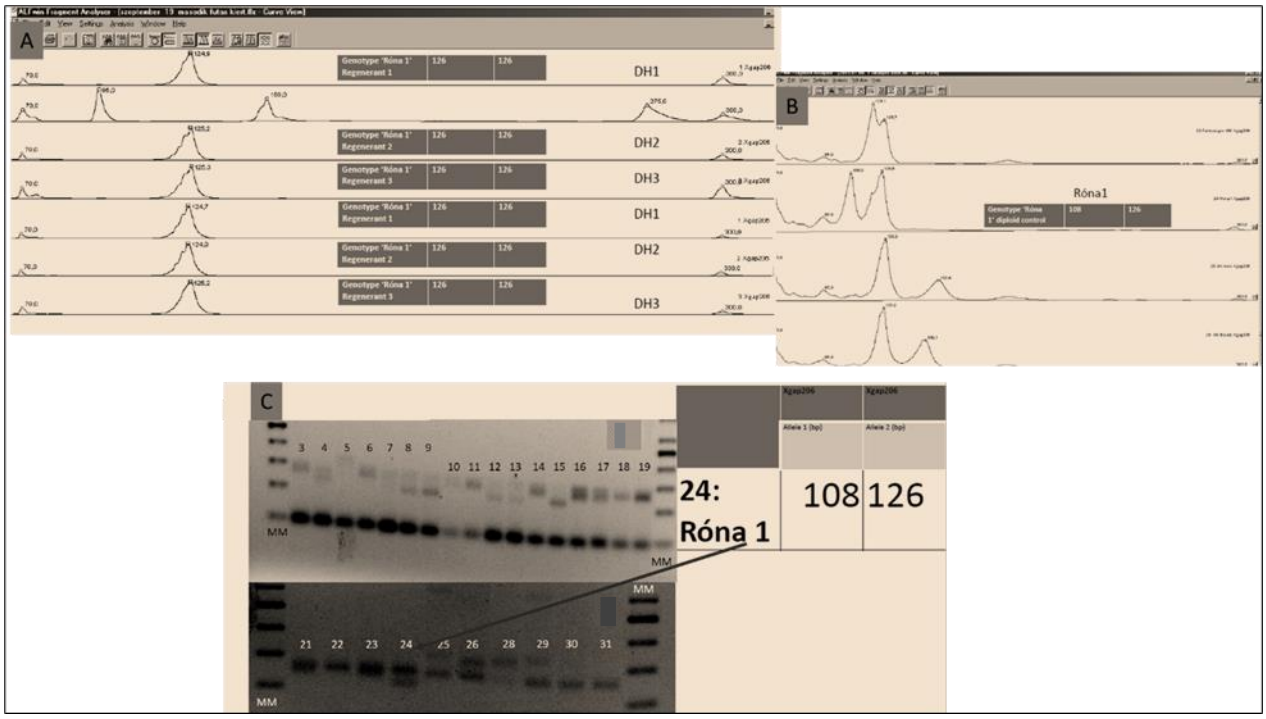

Figure 20. Gel images from molecular analysis of putative haploid regenerants of genotype Róna 1 and its diploid control with SSR primer mSbCIR238: A) Results from ALFExpress II DNA fragment analyzer machine showing just one allele (126 bp) for the 3 regenerants tested, B) PAGE results showing two allele sizes (108 and 126 bp) for the locus in the diploid control; C) Examined various sorghum diploid genotypes (3 to 31) on ethidium bromide-stained 2% NuSieveagarose gel **(**MM: Thermo Fisher Scientific Gene Ruler 50 bp Ladder) using primer mSbCIR238 with genotype Róna 1 (24) showing allele dimorphism characteristic for the diploid hybrid.

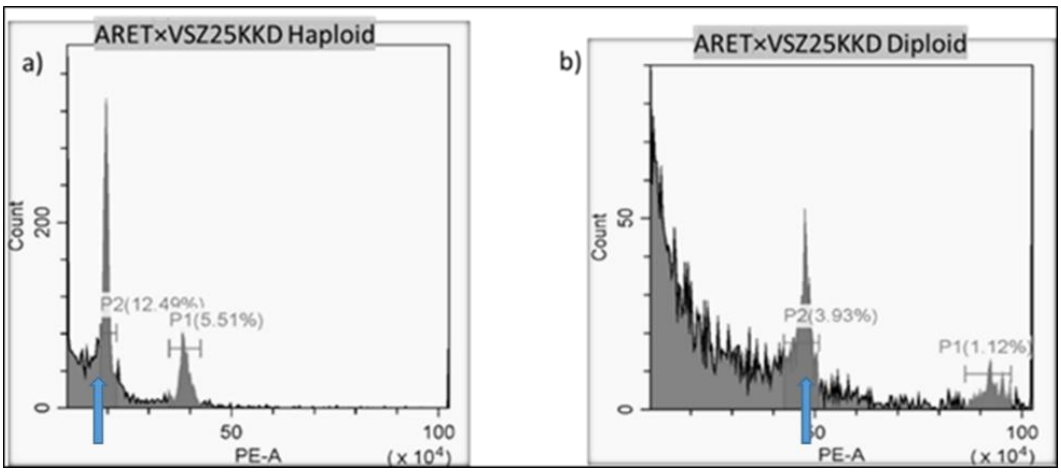

Figure 21. Flow cytometry histograms of sorghum F_1 experimental hybrid 'ARET×VSZ25KKD' haploids obtained (a) and their diploid control counterparts (b). P1 represents the DNA complement of the divided cell nucleus (cells at G2 phase of cell cycle) and their corresponding coefficient of variation (CV); P2 represents 'normal' haploid/diploid cells' (cells at G1 phase of cell cycle) DNA complement and their corresponding CV.

4.4.5 Chromosome duplication and determination of allele fixation

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, thus the fixation of alleles or not: 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. 22). The colchicine treated plants had an average of 144 grains per panicle, representing 9.03% of the total number of seeds per panicle.

Figure 22. 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.

5. DISCUSSION

5.1 Assessing the genetic diversity of some Selected Hungarian and East African Genotypes

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 (Tab. 18), 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.

Table 18. Genetic polymorphism parameters obtained with 5 SSR markers within 31 Sorghum genotypes

Locus	No. of alleles	Allele size range (bp)	Ho	He	PIC
Xgap-206	6	108 - 152	0.323	0.658	0.615
mSbCIR286	3	112 - 130	0.355	0.651	0.565
mSbCIR248	2	94 - 102	0.161	0.337	0.277
mSbCIR262	2	216 - 220	0.065	0.063	0.06
mSbCIR238	4	75 – 89	0.355	0.655	0.587
Total	17				2.104
Average	3.4				0.421

In the present study, the observed heterozygosity (Ho) was generally lower than the expected heterozygosity (He) in all the loci apart from mSbCIR262, 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 (mSbCIR262) to 0.615 (Xgap-206), with an average of 0.42 per locus. Marker mSbCIR262 was the least informative with a PIC value of 0.06 while marker Xgap-206 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. Since the SSRs used were co-dominant, there was detection of both alleles per locus. Allele frequencies were obtained by counting the proportion of each allele in the collection. The alleles at each locus and their frequencies are shown in Tab. 19. The allele frequency ranged from 3.23%, least to 96.77%, highest as follows: Xgap-206, 126 (54.84%); mSbCIR286, 112 (43.55%); mSbCIR248, 102 (79.03%); mSbCIR 262, 216 (96.77%); and mSbCIR 238, 75 (50%).

Table 19. Microsatellite loci, allele sizes in bp and their frequency in percentages among the sorghum genotypes

Fragment (bp)	**Frequency (%)**				
	Xgap-206	**mSbCIR286**	**mSbCIR248**	**mSbCIR238**	**mSbCIR262**
75				50.00	
81				16.13	
83				6.45	
89				27.42	
94			20.97		
102			79.03		
108	17.74				
112		43.55			
114	6.45				
120	9.68				
126	54.84	20.97			
130		35.48			
146	4.84				
152	6.45				
216					96.77
220					3.23

5.2 Suitability of 2,4-D and TDZ for somatic embryogenesis of sorghum [*Sorghum bicolor* (L.) Moench]

This study explored four callus induction media - half strength MS medium (Murashige and Skoog, 1962) supplemented with 2 mg/L2,4-D, 2 mg/LTDZ, 5 mg/L2,4-D and 5 mg/LTDZ labelled T1, T2, T3 and T4, respectively - for their effect on callus formation from apical/floral meristem explants of 10 sorghum hybrids. The results allowed us to determine the best growth

regulator between auxin 2,4-D and cytokinin TDZ and their best concentration for embryogenic sorghum callus induction. A quality callus in sorghum embryogenesis has been described variously as a white compact or creamy to yellowish (Tinak-Ekom et al. 2013). Furthermore, an EC contains nodules (Ko et al. 2008) which means it should be friable.

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 mg/L 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 mg/ L2,4-D, 1 g/L KH_2PO_4, 1 g/L L-asparagine, 1 g/L 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 mg/L 2,4-D. The trend changes on day 21, in conformity with the work of Assem et al. (2014) where they reported that 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. T2 and T4 which comprised half strength MS media supplemented with 2 mg/L TDZ and 5 mg/L TDZ, respectively, produced much lower EC% means compared with T1 and T3 at day 7 of the observations which significantly turned brown by day 35 (Fig. 14). Thidiazuron (TDZ) is described by Malik and Saxena, (1992) as a substituted phenylurea (N-phenyl-N-1,2,3-thiadiazol-5-ylurea) used as a synthetic herbicide and a plant growth regulator to stimulate high rate of axillary shoot proliferation in many woody plant species. Guo et al. (2015) notes that the molecule which was classified as a type of cytokinin due to its induction of many responses like those by natural cytokinins, has the ability for regenerative processes in cell and tissue cultures. While TDZ generated EC, there was high production of phenolic compounds in its presence, which led to the decline in EC% means by day 35. Although a higher TDZ concentration (5 mg/L) resulted in a higher level of phenolic exudates in the culture media, its EC% mean was not significantly different from that of 2 mg/L TDZ concentration.

Additionally, our study did not find EC (friability) induction to be influenced by genotype (Table 12). This in part contrasts with the findings of many studies which have reported that callus induction potential is influenced by genotypes for sorghum (Tinak-Ekom et al. 2013) and other crops such as rice as far as friability is concerned. The young inflorescence meristems from immature panicles were found to be effective sources of explants. It was established that 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.

5.3 Optimizing tissue culture media for sorghum somatic embryogenesis

This study explored the effect of six callus induction media (referred to in this study as types A, B, C, D, E and F) on their effect in generation of embryogenic callus from mature embryo explants of 10 Hungarian sorghum genotypes (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'). The study allowed an investigation on the difference in the effect of full strength and half-strength MS media modified with amino acids L-proline and L-asparagine, KH_2PO_4 and $CuSO_4 \cdot 5H_2O$ in sorghum somatic embryogenesis and to compare their efficiency in quality callus induction thereof with the induction on non-modified half strength MS, Chu N6, Gamborg B5 and 190-2 media. The information generated would provide clarity in sorghum somatic cultures, necessary for upstream processes such as doubled-haploid production, genetic transformation and others.

An embryogenic callus such as that described by Shireen et al. (2014) which is yellow, compact and friable is influenced by the medium type as opined by Tinak-Ekom et al. (2014) and the constituents of the media. 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 highlights 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 carried out on *Costus pictus.*

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 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 in the difference between 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. Our study provides the much-needed clarity in sorghum embryogenesis, regarding the effectiveness of MS based media as compared to non-MS types on sorghum calli induction, as well as the sensitivity of sorghum explants to the strength of MS media, making half strength MS media more effective in quality callus generation.

5.4 Doubled haploid production using an improved anther culture protocol for sorghum [*Sorghum bicolor* (L.) Moench]

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 haploid production, if the culture conditions were set at optimum. From the 28 different genotypes (both registered and experimental ones) in this study, 5 genotypes successfully produced haploids and subsequently four confirmed to be doubled haploid genotypes. This was a significant improvement compared to the works of Rose et al. (1986), Wen et al. (1991) and Kumaravadivel and Sree Rangasamy (1994). All of these authors 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.

The induction medium W14mf (Puolimatka and Pauk, 2000) differs from the other two media that were tested in the early phase of this study (summer of 2018) in that it contains $NH_4H_2PO_4$ (380 mg/L) and K_2SO_4 (700 mg/L) whereas the others do not. It contains disaccharide maltose (Blanc et al. 1999) as the carbon source at 80 g/L as compared to the 20 g/L sucrose in MS-t-z-2, while N_6 contains neither of the two. The other distinctive feature of W14mf is that it is a Ficoll®400 - a hydrophilic polysaccharide - supplemented medium devoid of a gelling agent, unlike MS-t-z-2 and N_6 which comprised of agar and Gelrite® as the gelling agents, respectively. It is noteworthy that according to Puolimatka and Pauk (2000) in their anther culture induction work on wheat (*Triticum aestivum* L.), the addition of Ficoll®400 or the use of maltose did not result in increased number of Embryo Like Structures (ELS) or that of the regenerable ones.

The use of W14mf medium resulted in the successful induction of four embryogenic calli for genotype 'GK Zsófia' in the summer of 2018, which regenerated into four haploid plants, whereas N_6 and MS-t-z-2 did not yield any. In the subsequent phases (winter of 2018/2019 and summer of 2019), only the W14mf was used, but this time involving modifications with KH_2PO_4, L-proline and L-asparagine, namely M1, M2 and M3 as earlier outlined. In the winter of 2018/2019, another genotype, 'Róna 1' produced five calli when induced on medium M2, of which only one regenerated, producing more shoots that were subsequently cloned into five haploid plantlets. In the year of 2019, a total of 52 calli from 9 genotypes; 21 calli on induction medium M2, 17 calli on induction medium M3 and 14 ones on induction medium M1 were obtained. Several researchers have reported that addition of the components L-proline, L-asparagine and KH_2PO_4 have led to the maintenance of yellowish colour of the calli and contributed to reduced production of phenolics in sorghum somatic tissue culture and higher callus induction rates (Liu et al. 2015; Sudhakar et al. 2009; Chege et al. 2020b). This was evident as medium M2 and M3 produced higher total number of calli (26 and 17, respectively) compared to the fourteen produced by M1 in 2019. It is however important to note that there were no significant differences observed in the callus induction among the control W14mf medium (M1) and the modified versions of it i.e., M2 and M3. The results of the current study additionally agree with the observations of Lantos and Pauk (2016), Ouyang et al. (1989), and Karimzadeh et al. (1995) that the use of W14mf as an induction medium has the potential to yield a high number of embryogenic calli and in regeneration doubled haploids in a wide spectrum of germplasms.

While culture medium composition and growth regulators therein are vital for a successful anther culture induction, other factors such as the developmental stage of the microspores, temperature, starvation and other forms of pre-treatment stresses may be important in the reprogramming of the pathway from gametophytic- to sporophytic development (Zarsky et al. 1992, 1995; Tourav et al. 1996; Wedzony et al. 2009). In the current study, a long cool temperature stress pre-treatment - after collection of panicles - was not seen to have any effect on callus induction, therefore agreeing with the findings of Rose et al. (1986). 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 more effective than the synchronization of microspores' development effect realized by a long temperature pre-treatment. Similar to the work of Kumaravadivel and Sree Rangasamy (1994) where a heat shock of 35 ˚C for 12 hours followed by an incubation temperature of 25 ˚C was included in one of the treatments, a heat shock of 32 ˚C for 72 hours followed by an incubation temperature of 28± ˚C for all the treatments was applied.

A case for combining temperature pre-treatment with osmotic starvation has been advanced by several authors in barley and maize androgenesis (Hoekastra et al. 1997; Kasha et al. 2002; Jacquard et al. 2003, Wedzony et al. 2009), especially the use of 0.3M mannitol to improve androgenic efficiency (Nageli et al. 1999; Zheng at al. 2003). The use of 0.3 M mannitol had been deemed particularly desirable for this study on sorghum anther culture induction since as Kasha et al. (2001) in androgenesis of barley had pointed out, it appeared to cause a genotype independent induction. Genotype dependency is a big inhibitor of efficient callus induction in sorghum- and led to regeneration of green plants in a shorter time. However, as was observed in this work, the use 0.3 M mannitol for osmotic starvation pre-treatment in sorghum anther culture did not provide any advantage to callus induction and frequency.

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. Out of the 9 responding genotypes, the experimental hybrid 'ARET×VSZ25KKD' had the highest induction rate at 9% on medium M2 in the year of 2019. Embryogenic calli were induced at the rate of less than 2% in the winter of 2019/2020, therefore confirming the reproducibility of the induction medium. 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. Hybrid genotypes 'GK Zsófia', 'Róna 1', 'ARET×VSZ25KKD', 'AREL×SZE697/01' and 'AREL×ZSV04/30' out of the nine responsive genotypes later regenerated into plantlets, 'AREL×ZSV04/30' producing an albino regenerant in the summer of 2019. This study applied 190-2 medium (Pauk et al. 2003) as the regeneration medium following an earlier trial on its applicability in sorghum calli regeneration by Chege et al. (2020a). 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. According to our results, the ideal temperature for sorghum regeneration under light condition was 25˚C, which corroborated the findings of Kumaravadivel and Sree Rangasamy (1994). In the reproducibility study in the winter of 2019/2020 of the current study, 'AREL×ZSV04/30' which had regenerated an albino plantlet in the previous season developed a green plantlet when the phase from induction to regeneration was maintained below 7 days. These findings were therefore in agreement with those of Wen et al. (1991) who concluded that the regenerations of albino plantlets or roots only in a medium was greatly reduced when calli were transferred to a regeneration medium as soon as callus was observed, therefore reducing the phase in the induction medium. In addition, the current study arrived at a similar conclusion with Wen et al. (1991), that calli that formed roots before shoots had very dismal chances of developing into shoots and therefore regenerating into plantlets. Interestingly, Wen et al. (1991) argued that the regeneration of albino plantlets indicated that they may have successfully developed haploids from their work.

The ploidy level of the regenerants was determined through the direct method of flow cytometry. Having identified the haploids through flow cytometry, haploidy or dihaploidy was confirmed with SSR analysis with selected primers (Billot et al. 2013) showing heterozygosity (allele dimorphism) only in the maternal genotype. All the haploids were found to be monomorphic in the tested loci while diploids were dimorphic. In total, five haploid varieties comprising of at least four individual plants, a majority of which were clones obtained from the regeneration of many shoots from a single callus per genotype, were obtained.

While chromosome duplication was done for one of the individuals of the haploid clones of genotype 'Róna 1' through colchicine treatment, it was observed that the remaining three clones spontaneously diploidized and produced seeds at 80.5, 20.9 and 2.4%. The colchicine-treated individual produced seeds at 9.03%, indicating that it was a partially fertile plant. These seeds will be used in the same way as those from the spontaneously diploid plants. This was indicative that enough fertility was obtained by the spontaneous di-haploids, therefore nullifying the need for intervention in chromosome duplication.

6. CONCLUSIONS AND RECOMMENDATIONS

While the main focus of this study was the endeavor to successfully produce doubled haploids of sorghum [*Sorghum bicolor* (L.) Moench], experiments toward several other specific objectives were undertaken. One of those was the determination of the genetic relatedness of some selected Hungarian sorghum germplasms with some East African counterparts with a view of obtaininig information about transferrability of germplasms and technological information across the two regions. As already mentioned, sorghum production in Hungary as in several other European countries has been growing steadily over the last decade with reports indicating a 10% increase in the planted area over 2019. Considering climate change realities, such as drought in summer, sorghum - which is a C4 tropical cereal with remarkable stress tolerance - is attractive for provision of feed and industrial bioethanol raw material. Genetic diversity analysis using SSR markers for 31 genotypes (16 from a Hungarian breeding programme at Cereal Research Nonprofit Ltd., Szeged, and 15 from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT-Nairobi) would therefore contribute in sourcing and pyramiding of genes that may be considered valuable for improvement of agronomic traits such as genetic barriers against different biotic and abiotic stress. ICRISAT-Nairobi has a wide collection of accessions, both hybrids and landraces in its gene bank, and determining relatedness with the breeding material in Hungary would inform diverse parental combinations in segregating progenies for further selection.

To enable this, the trial was conducted using SSR markers published by CIRAD. Cluster analysis with the SSRs' data resolved the 31 sorghum genotypes into two major clusters at 16% similarity threshold. Two of the clusters comprised a mix of both Hungarian and East African genotypes. The observed homozygosity (Ho) was generally lower than the expected heterozygosity (He) in all the loci, implying evenness of alleles with high homogeneity since majority of materials under trial were hybrids. The SSR markers were, however, not highly informative with an average PIC value of 0.421 and could not sufficiently separate some genotypes. The low similarity value among some sorghum genotypes could indicate that there is a high level of genetic diversity among the genotypes from the two gene pools.

The other objective of this work was to optimize and adapt a somatic embryogenesis protocol for sorghum. One of the efforts towards was to attempt to resolve a point of divergence among many researches, where different levels of auxin 2,4-D and cytokinin TDZ would be used in the induction medium in sorghum somatic embryogenesis. To achieve this, modifications of half strength MS medium with 2 mg/L 2,4-D (T1), 2 mg/L TDZ (T2), 5 mg/L 2,4-D (T3) and 5 mg/L TDZ (T4) respectively were applied in the embryogenic callus generation of 10 selected Hungarian

sorghum germplasms, from their young inflorescence explants. The ideal medium modification would yield a friable, free from browning callus, a suitable candidate for sorghum regeneration. The explants on the half strength MS media containing the two levels of 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin and thidiazuron (TDZ) a cytokinin were incubated in a dark thermostat at 28 °C for 5 weeks. The number of viable calli that were embryogenic and yellowish/cream in color (EC) was recorded each week, alongside that of those which were embryogenic but brown (EB); non-embroygenic and cream (NEC); and non-embroygenic brown (NEB). The study recorded the highest mean of EC (62.17 ± 2.9) with T1, which was significantly different ($P < 0.05$) from the other treatments. The results from this first experiment on effects of different plant hormones on sorghum embryogenesis corroborates with earlier findings which demonstrated that a significant increase in 2,4-D had an inhibitory effect on callus yield. Reduced presence of sterile explant parts other than those containing intercalating tissues had a significant contribution to the reduction of lethal browning in the cultures. These results provide consolidated basic information on sorghum embryogenesis and this level of 2,4-D was later applied in all the induction media used for microspore culture in the doubled haploid production experiment.

On the other hand, this project considered that the use of appropriate culture media in somatic embryogenesis plays a crucial role in ensuring the induction of quality embryogenic calli, which assure subsequent efficient regeneration. This was in the endeavour to optimize and adapt somatic embryogenic protocols. The project therefore assessed the suitability of various induction media to produce quality callus with high induction rates, that suppresses the production of phenolics (lethal browning). Six media types: 3 based on MS media and one each based on N6, Gamborg B5 and various modifications of both MS and N6 culture media were applied in the embryogenic callus generation in 16 selected Hungarian sorghum germplasms from their sterile seed-started plantlet explants.

The ideal media modification would yield a friable callus free from browning, a suitable candidate for sorghum regeneration through indirect organogenesis. Although, there was a genotype influence on the attainment of a yellow callus, friability of the callus was determined to be dependent on the culture medium and not the genotype. Half strength MS medium with 0.2 mg/L 2,4-D with 2.8 g/L Gelrite® as the gelling agent modified with 1.0 g/L KH_2PO_4, 1.0 g/L L-proline, 1.0 g/L L-asparagine and 0.16 mg/L $CuSO_4 \cdot 5H_2O$ (type E) was found to be the most effective resulting in about 60% yellow coloured callus induction with 25% friability. Addition of $CuSO_4 \cdot 5H_2O$, KH_2PO_4, L-proline and L-asparagine significantly reduced the phenolic production in sorghum somatic culture on MS media. Half strength MS medium was observed to contribute

to quality callus production when compared to full strength MS media modified with the compounds. The half strength MS medium was also observed to suppress phenolic production. The results provide information on a suitable sorghum callus induction medium necessary for embryogenesis.

The major focus of this project was however on describing an optimized protocol for sorghum doubled haploids through the regeneration of sorghum doubled haploids at F2. This idea was anchored on the belief that sorghum, like other major cereals can benefit from accelerated breeding and release of improved varieties through doubled haploid technology. The project therefore envisaged overcoming the major drawback that hitherto greatly hampered this endevour, namely, the lack of a reproducible protocol for technology on the crop, that can overcome genotype dependency and other species-specific challenges such as phenolic exudation. This study aimed at regenerating sorghum haploids and diploidize them to achieve genetic and phenotypic homozygosity, thereby contributing to the development of an improved protocol.

From the 28 hybrid genotypes, both F1 registered- and experimental hybrids involved, this study successfully produced haploids from five varieties and subsequently, four 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. Cold temperature and starvation pre-treatment were not found to provide any advantage to callus induction. Medium 190-2Cu was used for regeneration and rooting, which occurred successfully, if the calli were transferred on to it less than 7 days after induction, and temperature was maintained at 25˚C under light condition. While genotype dependency was not wholly overcome by this protocol, sorghum's high tillering ability and high abiotic stress tolerance were observed to greatly contribute to attainment of haploid plantlets. The crop was found to spontaneously diploidize and produce seeds at rates of upto 80.5%, therefore eliminating the need for colchicine treatment for chromosome duplication.

7. 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.

8. SUMMARY

Sorghum [*Sorghum bicolor* (L.) Moench] is an important staple cereal crop, especially in the sub-Saharan Africa. Several authors describe the crop as an important food crop to the diets of the poorest of the world's population. This is occasioned by the fact that sorghum is a drought tolerant C4 grass, which provides grain yields when other crops have failed due to droughts in the rainfed systems. Sorghum is, however, not just a preserve of the sub-Saharan Africa, recently gaining recognition and preference in the diets of northern Europe, where celiac disease is most prevalent. This is because sorghum has been found to provide protein digests that are safe and tolerable to people suffering from this disease. These, among other reasons such use as feed and in industrial processing, makes sorghum fit the description, the grain of the future as some authors have called it.

It was this new interest in sorghum among growers in Europe and especially Hungary that informed this project's decision to determine the genetic diversity that exist between the germplasm that is currently grown in Hungary and those from sub-Saharan Africa (East Africa). Molecular markers (SSRs) were employed for this purpose and it was established that there was a high level of genetic diversity among genotypes in the two regions. Following this, the project therefore finds it favourable for the transfer of genetic material and knowledge concerning new technologies on the crop, developed in either of the regions to be enhanced.

The main focus of this project was however on the development of an optimized protocol for production of sorghum doubled haploids. Sorghum, which is the 5th of the most consumed cereals globally, like the other major cereals would also benefit from the development of this technology. It has been severally reported that protocols for sorghum doubled haploid production remain unavailable or unreproducible. Doubled haploid 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. The realization of this would enable the breeders to address the challenges of yield reducing factors such as pests susceptibility, therefore availing the much needed food, feed and industrial raw material that sorghum provides in a short period of time.

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 callus 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 induction compared to the synchronization of micropore maturation effect of pre-treatement 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%.

Concerning the project's effort to optimize and adapt somatic embryogenesis protocols, it 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 15 day old germinating embryos. The addition of components L-proline and L-asparagine, KH_2PO_4 and $CuSO_4 \cdot 5H_2O$ contributed significantly to maintaining creamy colour of the calli. Furthermore, the project found that phenolic exudation was greatly reduced when the 1.5 day old germinating but undifferentiated embryos (intercalating tissues) were used as explants, instead of already differentiated tissues such as stems and leaves. The endeavor to optimize and adapt sorghum somatic embryogenesis protocols also established that 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.

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11. Appendix

Appendix 1: Stock genomic DNA concentration and normalization in 31 sorghum genotypes

S/No	Name	Nano drop reading ng/µl	DNA µl	H2O µl to dilute to 10 ng/µl
1	ICSR9303	60.53	8.26	41.74
2	Framida	37.00	31.51	36.49
3	Seredo	30.50	16.39	33.61
4	Hakika	112.88	4.43	45.57
5	EC-Teso	65.20	7.67	42.25
6	Mahube	35.60	14.04	35.96
7	KARI Mtama 1	31.50	15.87	34.13
8	Abaleshya	25.50	19.60	30.40
9	IESV91054LT	33.60	14.88	35.12
10	F6YQ212	79.88	6.26	43.74
11	Gadam el Hamam	90.00	5.55	44.45
12	Cytanombe	62.20	8.04	41.96
13	IESV900I5LT	78.00	6.41	43.59
14	IESV91069LT	43.70	11.44	38.56
15	Ndamoga	76.20	6.56	43.44
16	Alföldi 1	38.10	13.12	36.88
17	GK Emese	18.50	27.03	22.97
18	GK Zsófia	27.90	17.92	38.08
19	SREZA× (A119 ×	13.40	37.31	12.69
20	ARET×VSZ21KKD	18.20	27.47	22.53
21	AREL×VSZ21KKD	21.80	22.93	27.07
22	A119 × SZeTC73	45.00	11.11	38.89
23	Farmusugro 180	22.70	22.03	27.97
24	Róna 1	28.60	17.48	32.52
25	GK Áron	17.00	29.41	20.59
26	GK Balázs	15.80	31.65	18.35
27	GK Erick	20.60	24.27	25.73
28	(A119×KS61B)	13.50	37.04	12.96
29	(A119×KS60B)	13.50	37.04	12.96
30	AIL-1× B119×Va-	15.30	32.68	17.32
31	GK Csaba	12.80	39.06	10.94