Chapter ONE

Introduction

1.1 The sorghum plant

1.1.1 Sorghum - Origin and history

Sorghum bicolor L. Moench (2n = 20) ranks fifth in commercial production among cereals in the world; in order of commercial production, sorghum is behind the following cereals: rice, maize, wheat and barley. It is believed that sorghum originates in Sub-Saharan Africa and the crop is well adapted to the semi-arid and subtropical conditions of this area. However, the distribution of the genus *Sorghum* is world-wide. There is strong belief that this was due to the continental drift of the Pangaea that took place more than 220 million years ago (Doggett, 1988). There is a strong belief that this crop originally belonged to the Congolese tribe called Andropogoneae who lived in the tropical regions of Africa. Three species were identified, i.e. *S. bicolor*, *S. halepense* and *S. propinquum*. Different classification systems for sorghum were provided by a few authors (Harlan and De Wet, 1972; De Wet, 1978). An in-depth review of the three species was provided by De Wet (1978). These classification systems included observation for different shapes, sizes and colour of the grains, glumes, panicles, leaf blades and the whole plant. *S. bicolor* is an annual plant that forms tillers, with an erect plant stature, ranging from 0.5 m to over 5 m in height and with long leaf blades up to 90cm and 12cm wide. The fruit forms either an open or closed panicle that is 5-60cm long and 3-30cm wide.

A B

Figure 1.1 Sorghum plants cultivated in the greenhouse at the CSIR Biosciences, Pretoria, South Africa. A: Full-grown plants. B: Panicle with developing seeds.

The cultivated sorghum of today is strongly believed to have arisen from the wild *Sorghum bicolor* subspecies *arundinaceum* (Mann *et al*., 1983). The Ethiopian and Sudanese Sorghum Conversion Programs possess the widest range of sorghum germ-plasm database and this further indicates the origin of the crop. This also suggests that human selection in these areas has been in existence for a very long time.

1.1.2 Agronomic attributes

1.1.2.1 *Vegetative morphology*

S. bicolor is an annual plant that forms tillers, has an erect plant stature and can grow up to 5 m in height. The stem is generally hardened but the centre is usually spongy. The lowest nodes form roots and the tall varieties have roots higher up the lowest node. This ensures a good

support structure for the plant, e.g. against strong winds. The growth rings that usually form at each node are capable of differentiation when needed, e.g. when the plant has been floored by strong winds, an upright position may be regained through these growth rings. Each node also possesses a single bud that can either develop into tillers or branches when required. The branches and tillers usually form as a result of stress caused by stem borers or other environmental damage (Doggett, 1988).

Tillers generally form as a way of plant survival. Tiller formation in sorghum is subject to variety; certain varieties tiller early while others tiller post flowering. Temperature and photoperiod also play a role in tiller formation with lower than 18°C temperatures promoting formation of tillers while short photoperiods do not. These tillers enable certain varieties to survive for a number of years (Duncan *et al*., 1981).

The number of leaves may vary from 7-24 and mature leaves vary between 30-135 cm in length and between 1.5–13cm in width. Protection against shoot-flies is conferred by the presence of trichomes (prickle hairs) on the abaxial surface of the leaves. During drought stress, motor cells enable the leaves to roll inwards. Sorghum also has an adventitious root system with a number of branched lateral roots and they can extend up to 1.5 m deep into the soil. This enables very good nutrient absorption.

1.1.2.2 *Temperature*

There are three classified groups of sorghum based on their adaptation to temperature, i.e. cold-tolerant, temperate and lowland tropical sorghum. The cold-tolerant sorghums have the ability to

tolerate both day and night cold temperatures of 17°C and below. They are also known as high-altitude sorghum because of their general ability to grow on elevated landscapes. In the USA, numerous studies have concentrated on the temperate sorghum due to substantial interest in its development. This group can tolerate cold temperatures both at the initiation and at the end of the growing season with the exception of the flowering period. They grow best during very warm days and cool nights (Doggett, 1988). The lowland tropical sorghum prefers both warm days and warm nights (25-34°C) throughout the growing season. They are also best suited for lower altitudes.

1.1.2.3 *Physiology*

The genotype-environmental relationship involving the interactions of environmental factors on the genetic make-up of a plant often selects for the physiology and subsequent product or phenotype. Conversely, this means that the optimum cultivation conditions for a particular plant select for that particular plant against others. Temperature and photoperiodicity are two important abiotic factors that influence this relationship.

1.1.2.4 *Photoperiodicity*

The cold-tolerant and lowland tropical sorghums are photoperiod sensitive while the temperate sorghum is not (Doggett, 1988). The studies on the temperate sorghums revealed a strong interaction between genotype, photoperiod and growing temperature. The time of flowering was significantly affected by this relationship (Miller *et al*., 1968).

1.1.3 Cultivation advantages and soil pH

Sorghum has the ability to better withstand drought conditions and tolerate a wider range of soils than other cereal crops. Due to a lack of resources in these areas, farmers in the developing world are usually forced to grow cultivars with the ability to produce under harsh conditions that include drought stress, pests and disease. The pH range suitable for growth is 5.5-8.5. Tolerance to poor drainage and saline soils has been displayed as well (Doggett, 1988).

1.2 **Grain utilization**

In the developed world, sorghum is grown mainly for grain and forage mostly to feed livestock, but the developing world uses sorghum products as staple food. Millions of people in Africa and Asia process and consume the grain in various ways. The plant stems can also be used for brooms and in the sweet varieties, they are chewed and processed into other products due to the high sugar content. More recently, the sweet-sorghums have become increasingly important as a potential source of ethanol for biofuel. Grain usage includes the following:

- traditional home-made meals,
- immature grain foods,
- boiled grain meals,
- roasted grains,
- popped grains,
- fermented meals,
- sorghum breads,
- stiff porridges, and
- sorghum beers.

1.3 Genetic engineering of plants

Plant genetic engineering is the application of recombinant DNA technology in plants to study and introduce foreign genetic material. This technology also focuses on the interface of all aspects of cell biology and molecular biology. The problems faced by plant breeders, such as costly and prolonged breeding methods, have led to technologies that were designed to both assist and solve plant breeding obstacles. The low probability of achieving a product that displays multiple foreign phenotypic characters gave birth to the establishment of genetic engineering tools to facilitate precision insertions of specific multiple traits in a desired genetic background. These include recombinant DNA technology, *in vitro* tissue culture and plant transformation, somaclonal and gametoclonal variation.

The first genetically modified species was the bacterium *Escherichia coli* in 1973 (Cohen *et al*., 1973) that expressed a *Salmonella* gene. The development of the methods to extend the principle of inter-species gene expression began in 1983. In the midst of ethical and complex issues surrounding the production of genetically engineered (GE) plants, transgenic crops production has since become a major scientific research and commercial component. This is supported by the data in Table 1.1, wherein the transgenic crops take up a significant fraction of the total planted land in each of the top 18 countries that grow these GE crops.

The major GE crop varieties commercialized since 1996 have been designed to help control insects using *Bacillus thuriengensis* toxins and herbicide-based weed management systems using glyphosate. The genetic modification of crops have also been accomplished in fruits such as

banana (Ságí *et al*., 1994) and citrus (Vardi *et al*., 1990); in trees such as *Casuarina glauca* Sieb. Ex Spreng (Smouni *et al*., 2002); and in beverages such as coffee (Perthuis *et al*., 2005).

Table 1.1 Worldwide status of transgenic crop production - 2005

Rank	Area	Area (mil. Ha/A.)	Crop
1	USA	49.8/723.0	soybean, maize (corn), cotton, canola, squash, papaya
2	Argentina	17.1/42.2	soybean, maize, cotton
3	Brazil	9.4/23.2	soybean
4	Canada	5.8/14.3	canola, maize, soybean
5	China	3.3/8.2	cotton
6	Paraguay	1.8/4.4	soybean
7	India	1.3/3.2	cotton
8	South Africa	0.5/1.2	maize, soybean, cotton
9	Uruguay	0.3/.7	soybean, maize, cotton
10	Australia	0.3/.7	cotton
11	Mexico	0.1/.2	cotton, soybean
12	Romania	0.1/.2	soybean
13	Phillipines	0.1/.2	maize
14	Spain	0.1/.2	maize
15	Colombia	<0.1/.2	cotton
16	Iran	<0.1/.2	rice
17	Honduras	<0.1/.2	maize
18	Portugal	<0.1/.2	Maize

1.4 Genetic engineering of sorghum

A suggestion was aired by Birch and Bower (1994) that one of the most important aspects for successful particle bombardment in generating transgenic plants was an efficient selection for transformants. In sorghum, for a long time, the selectable marker gene *bar*, an herbicide resistance gene, was used in most sorghum transformation reports. The *hpt* (hygromycin phosphotransferase) gene was only used once by Hagio (1991). The *pmi* (phosphomannose isomerase) gene was only recently reported by Gao *et al*., (2005).

1.4.1 *In vitro* regeneration of cereals

Cereals, woody trees and legumes were classified as highly resistant to *in vitro* manipulation and tissue culture regeneration until 1982 (Barton *et al*., 2000, patent). Although callus cultures of rice (*Oryza sativa*) were first achieved in 1964 (Oono, 1983), there were no reports of reproducible and sustained production of fertile and normal plants of genotype. A number of breakthroughs took place in the early 1980s and these formed the basis for the regeneration of almost all the cereals by *in vitro* tissue culture.

The breakthroughs were as follows: (a) For *in vitro* tissue culture, immature embryos, young inflorescences and the bases of immature leaves can regenerate into fertile plants; (b) this process involves a simple nutrient medium with high concentration of a strong auxin like 2,4-dichlorophenoxyacetic acid (2,4-D); (c) plant regeneration takes place mainly through somatic embryogenesis; and (d) totipotent protoplasts are a result of embryogenic cell suspension cultures derived from embryogenic calli. A number of factors influence the tissue culture

amenability of cereals, i.e. choice of explant, medium composition, culturing and regeneration conditions, and these were elucidated (Vasil, 1994; Vasil and Vasil, 1992, 1994).

1.4.2 *In vitro* regeneration of sorghum

The first sorghum tissue culture and plant regeneration was reported by Masteller and Holden (1970). High frequency plant regeneration from cultured explant material is crucial for the successful transformation of most cereal crops (Rachmawati and Anzai, 2006).

1.4.2.1 *Explant*

The choice of explant has been identified as one of the most important factors for cereal *in vitro* regeneration - this includes the physiological and developmental state of this plant material. In sorghum, immature zygotic embryos (Gamborg *et al*., 1977; Thomas *et al*., 1977; Cai *et al*., 1987; Dunstan *et al*., 1978, 1979; Brar *et al*., 1979; Ma and Liang, 1987), mature embryos (Thomas *et al*., 1977; Cai *et al*., 1987), immature inflorescences (Brettell *et al*., 1980; Boyes and Vasil, 1984; Cai and Butler, 1990; Kaeppler and Pederson, 1997), seedlings (Masteller and Holden, 1970; Brar *et al*., 1979; Davis and Kidd, 1980; Smith *et al*., 1983), leaf fragments (Wernicke and Brettell, 1980) and anthers (Rose *et al*., 1986) have been used as explants. In cereals, the immature zygotic embryos' scutellum tissue has been a reliable target for somatic embryo formation and, hence, easier uptake of DNA for generation of transgenic plants, e.g. in barley (Wan and Lemaux, 1994), in maize (Brettschneider *et al*., 1997), in oat (Somers *et al*., 1992), in rice (Christou *et al*., 1991), in rye (Castillo *et al*., 1994), and in wheat (Vasil *et al*., 1992). In sorghum, immature zygotic embryos or the callus derived from them has been an

explant of choice (Casas *et al*., 1993; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002; Gao *et al*., 2005).

1.4.2.2 *Media composition*

In vitro culture of cereals shows strong genotype dependence and production of the ideal cultures is generally limited to selected genotypes. The first stable transgenic sorghum plants were regenerated on callus initiation medium containing MS salts (Murashige and Skoog, 1962), modified (without calcium pantotenate) B5 vitamins, and 8g/l agar (Casas *et al*., 1993). This was supplemented with asparagines (150µg/ml), 10% coconut water, 30g/l sucrose and 2mg/l 2,4-D. For several sorghum genotypes tested in the CSIR's plant biotechnonolgy laboratory (Pretoria, South Africa), the medium based on Casas' yielded very low regenerants per explants values – the value calculated by dividing the number of individual fertile plants by the number of explants cultured on initiation medium (data not published).

1.4.3 Sorghum transformation

Among the direct gene transfer methods used in cereals, four are reliably used routinely to produce transgenic plants, i.e. protoplast transformation, tissue/cell electroporation, silicon carbide fibre vortexing and particle bombardment, also known as biolistics transformation. For sorghum, there are mainly two approaches currently in use for introducing foreign genes in cereals. The first one, called particle bombardment or biolistics, involves a physical method to transfer genes into target tissue. The second method, *Agrobacterium*-mediated transformation,

involves a natural plant pathogenic bacterium (*Agrobacterium tumefaciens)* that is genetically modified to transfer a segment or segments of DNA into plant cells.

In cereals, much of the early transformation methods were limited to direct gene transfer techniques because early work using the *Agrobacterium* failed to produce stable transgenic cereal crops (Shewry *et al*., 2001). However, Zhao *et al*., (2000) were the first to report on stable sorghum transgenic plants using the *Agrobacterium* method. In sorghum, the *Agrobacterium*-mediated transformation has recently been a preferred method of choice over biolistics. This is mainly due to simpler integration events that this method tends to yield and the perceived lower transformation efficiencies by biolistics in comparison to *Agrobacterium*-based methods (Table 1.2). However, Table 1.2 provokes an argument over this perceived superior transformation efficiency of the *Agrobacterium*-based method. This is because the transformation efficiencies are closely comparable. Using biolistics, Girijashankar and colleagues (2005) reported a superior value of 1.5 over Howe and colleagues' 1% (2006), who used the *Agrobacterium* method. Furthermore, no comparison of the two methods' transformation efficiencies has been reported in sorghum.

However, particle bombardment has also been used to successfully produce one of the most commercially important transgenic events called the *Mon810* or Yieldgard in *Zea mays L.* (maize). This transgenic event confers resistance to European Corn Borer (*Ostrinia nubilalis*) through the expression of a *Bacillus thuringiensis* Cry1AB protein (Sanders and Patzer, 1995). This transgenic event displayed a simple integration of the transgene (one copy), suggesting that

biolistics can also be used in sorghum for the production of transgenic plants with agriculturally important traits.

Sorghum has been classified as a recalcitrant cereal with regards to tissue culture regeneration and transformation (Kresovich *et al*., 1987; Zhu *et al*., 1998). It is ranked lower than important cereals such as maize, rice, wheat and barley and as such has not been a priority for improvement using biotechnological techniques.

Table 1.2 List of stable sorghum transformation reports to date.

Transformation method	**% Transformation efficiency (# expl.)**	**Authors**
Biolistics	0.08	Casas *et al*., 1993
Biolistics	0.33	Casas *et al*., 1997
Biolistics	0.09	Zhu *et al*., 1998
Biolistics	1.00	Able *et al*., 2001
Biolistics	0.18	Emani *et al*., 2002
Biolistics	1.30 (375)	Tadesse *et al*., 2003
Biolistics	1.5 (200)	Girijashankar *et al*., 2005
Agrobacterium	2.10 (6175)	Zhao *et al*., 2000
Agrobacterium	2.50 (2463)	Gao *et al*., 2004
Agrobacterium	2.91 (310)	Gao *et al*., 2005
Agrobacterium	1.00 (4511)	Howe *et al*., 2006

Selection for sorghum transgenic tissue after particle bombardment is crucial because the mechanical damage on explants requires careful *in vitro* culturing to recover and regain tissue totipotency. Bialaphos is a tripeptide antibiotic, which consists of the active ingredient phosphinothricin (PPT). PPT is a glutamate analogue and possesses the inhibiting effect on the enzyme glutamine synthetase (GS). This enzyme is responsible for detoxifying ammonia accumulation to toxic levels in cells, thus the inhibition effect of GS leads to tissue death. The *bar* gene encodes the enzyme phosphinothricin acetyl transferase (PAT) that detoxifies PPT by acetylation (D'Halluin *et al*., 1992). In contrast, the *pmi* selection system is considered a more tissue friendly system as the transgenic tissue is privileged with the ability to metabolize a different carbon source. Therefore, the *pmi* selection system's superiority over the *bar* selection system is believed to be attributed to its mode of action. In short, the *bar* system aims at killing cells while the *pmi* system starve the non-target tissue while promoting cell proliferation of the target cells.

1.5 Nutritional quality of the grain

Cereal grains are among the most important dietary sources of proteins, carbohydrates, B vitamins and minerals in the world. The nutritional quality of the sorghum grain is determined by the content of its constituents, i.e. proteins, carbohydrates, and lipids. The biological processing of proteins from any dietary source depends on their amino acid composition, secondary structure and the interactions of such proteins with any other matrices (tertiary and quaternary structures) which have implications for bio-digestibility and overall nutritional value. For all cereal grains, lysine is the first essential amino acid that is limiting for the diets of humans and monogastric animals (Table 1.3). Because of this, sorghum seed proteins are only partly used as

energy source in feed. An excess of certain amino acids combined with a shortage of others may result in deleterious effects of certain proteins that are essential for metabolic processes (Eggum *et al*., 1981).

The *Sorghum* seed has a nutritional profile similar to maize and other cereals (Shewry and Halford, 2002). The other major factor that limits sorghum nutritional quality is the seed protein digestibility. Sorghum seed protein digestibility, in comparison with other cereals, decreases significantly upon wet cooking (Duodu *et al*., 2002). This is attributable to the disulphide bond formations between protein polymers that contain sulphur in their side chains, e.g. cysteine (Hamaker *et al*., 1987; Rom *et al*., 1992; Duodu *et al*., 2003). Oria and colleagues (1995b) cited the cysteine-rich β- and γ- kafirins cross-linkages with themselves and with other matrix proteins as responsible for lowered protein digestibility. Granted that this postulation was accurate the β- and γ- kafirins, which are mainly on the periphery of the protein bodies, form a barrier that renders the more abundant α-kafirins on the inside unreachable by proteolytic enzymes such as pepsin.

A general structural organization of the sorghum seed was thoroughly reviewed (Hoseney, 1994). The generalized longitudinal structure is displayed in Figure 1.2. The estimated distribution by weight is generally as follows: endosperm - 84%, embryo/germ - 10% and pericarp - 6%.

Table 1.3 Sorghum kafirin amino acid content (mole %).

Amino acid	α-Kafirin	β-Kafirin	γ-Kafirin	Total Kafirin
Asparigine	6.0	3.3	0	4.8
Aspartic acid	0.4	N/A	0	N/A
Threonine	4.0	4.6	4.7	2.8
Serine	6.0	4.6	5.2	4.7
Glutamine	24.6	17.8	11.9	20.0
Glutamic acid	0.4	N/A	1.0	N/A
Proline	7.7	9.7	23.3	11.2
Glycine	1.6	6.8	8.8	2.7
Alanine	14.9	13.4	5.7	15.6
Cysteine	0.4	4.9	7.8	0.7
Valine	4.4	5.2	6.2	5.6
Methionine	0.8	5.7	1.0	1.7
Isoleucine	5.6	2.3	2.6	4.1
Leucine	15.3	12.0	8.3	15.4
Tyrosine	2.8	3.0	2.1	3.0
Phenylalanine	2.4	1.9	1.6	4.7
Histidine	1.2	0.9	7.8	1.6
Lysine	0	0.5	0	0.2
Arginine	0.8	2.7	2.1	1.2
Tryptophan	0.4	N/A	0	N/A

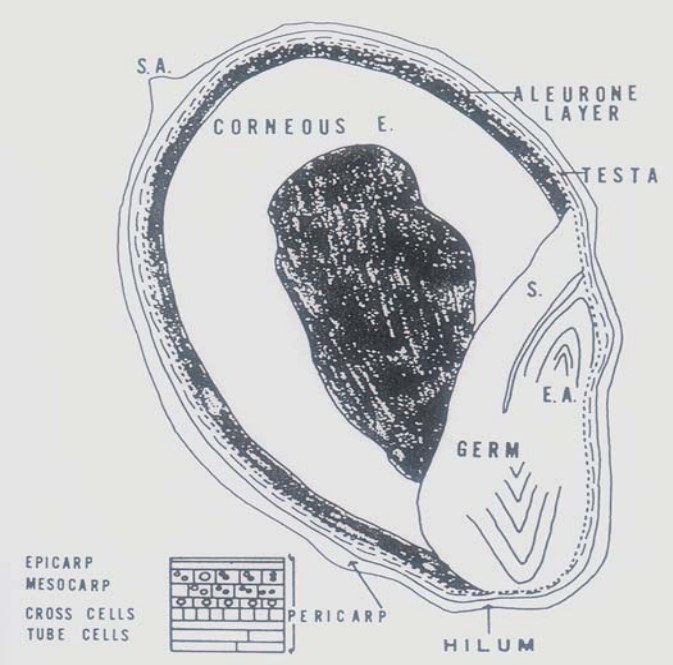

Figure 1.2 The longitudinal cross-section of the sorghum seed structure (Hoseney, 1994).

1.5.1 Seed carbohydrates

Starch is a major carbohydrate in sorghum and an average of 73% per seed dry weight is recorded (Olson and Frey, 1987). Starch occurs in granules which are both spherical and polygonal. These granules vary from 4-24µm in diameter. While 21-34% of this starch is amylase, the rest is amylopectin. Altogether 1.2-5.2% cellulose is mainly accounted for in the pericarp.

1.5.2 Seed protein

Seed protein content ranges from 7-14%. Most of the protein that accumulates during seed development is in the form of storage protein, but a small pool of amino acids is free amino acid

which account for about 10% or often less of the total amino acid content. The storage proteins have the following characteristics:

- they have no enzymatic activities,
- their synthesis is mainly during seed development,
- they are packaged in spherical structures called protein bodies, and
- they provide nitrogen, carbon and sulphur for the germinating seedling.

The content of storage proteins determines the seed quality and end use properties. Most variations are caused by environmental effects. Because the endosperm is a major part of the seed, its composition determines the seed nutritional quality. Two major types of storage proteins occur in seeds, i.e. the globulins and the prolamins. Globulins predominate in dicotyledons, but also in the aleurone layers and embryo of cereals, while prolamins are only found in the endosperm of all cereals.

1.5.2.1 *Sorghum storage globulins*

The globulin storage proteins are soluble in dilute saline solutions and are mainly packaged and stored on the embryo and aleurone layer. These storage proteins are rich in asparagines and glutamine and very poor in the sulphur-containing amino acids cysteine and methionine. Methionine is one of the essential amino acids and when it is limiting in diets, cysteine becomes essential because it is synthesized from methionine.

Two structural groups of storage globulins make up most of the globulins. These are distinguished by their sedimentary co-efficiency of 7S and 11-12S. The 7S globulins appear to

function only as protein bodies and are not involved in normal seed function. This was evident in maize where a mutant lacking these proteins displayed normal germination and general seed development (Kritz and Wallace, 1991). In comparison with the starchy endosperm, the embryo and the aleurone layer are richer in protein content but these proteins do not make a difference on the end usage in terms of seed quality. This is because they are usually lost during milling (wheat), polishing (rice), pearling (barley) and decortication in sorghum (Shewry and Halford, 2002).

1.5.2.2 *Storage prolamins*

The prolamins of sorghum are also called kafirins while in maize, a close relative of sorghum, they are called the zeins. They are rich in amino acids proline and glutamine but deficient in lysine, tryptophan and threonine. Based on their alcohol solubility, the kafirins were initially divided into 4 groups, i.e. α-, β-, γ- and δ-kafirins (Shewry and Halford, 2002). However, although some prolamins occur in alcohol-insoluble polymers, they are all alcohol soluble in a reduced state.

The most abundant α-kafirins are mainly polypeptides of 19-24kDa. These kafirins contain one or two cysteine residues per molecule, much less than the other four minor groups. The β-kafirins are polypeptides of 14-16kDa that are lower in proline and glutamine than the α-kafirins. These kafirins are rich in proline and cysteine. Two major γ-kafirins are polypeptides of 27 and 16kDa, while a minor γ-kafirin is 50kDa in size (Xu and Messing, 2008). The δ-kafirins are a minor fraction of 10 and 18kDa polypeptides that are extremely rich in sulphur-containing amino acid cysteine.

The kafirins are believed to be synthesized in the rough endoplasmic reticulum (ER) and transported from the ER via the Golgi apparatus and into the protein storage vacuole (Taylor *et al*., 1985; Kermode and Bewley, 1999), but the actual mechanism is still not well understood. However, a number of reports attempting to elucidate this mechanism in species such as maize (Coleman and Larkin, 1999; Muench *et al*., 1999), rice (Yagamata and Tanaka, 1986; Krishnan *et al*., 1986), oats (Lending *et al*., 1989), and wheat and barley (Galili, 1997; Shewry 1999) have been provided, therefore, more studies should focus on clarifying this process to fully understand the stereo-chemistry of protein bodies.

1.5.2.3 *Dietary lysine*

Lysine is one of the ten essential amino acids for mammals, with the molecular formula $C_6H_{14}N_2O_2$. Its three and one letter codes are Lys and K respectively, while the nucleotide translation codons are AAA and AAG. Table 1.4 lists the ten essential and ten non-essential amino acids for mammals. In plants and micro-organisms, lysine is synthesized by a complex cycle that involves aspartic acid as a substrate (Figure 1.3). Methionine and threonine are also synthesized by this pathway (Sakai *et al*., 2003; www.genome.ad.jp/kegg/pathway/map/map00300.html).

Table 1.4 List of 10 essential and 10 non-essential amino acids in mammals

Essential amino acids	**Non-essential amino acids**
Arginine	Alanine
Histidine	Asparagine
Isoleucine	Aspartate
Leucine	Cysteine
Lysine	Glutamate
Methionine	Glutamine
Phenylalanine	Glycine
Threonine	Proline
Tryptophan	Serine
Valine	Tyrosine

The daily lysine nutritional requirement in humans is 1-1.5g and Table 1.5 shows a comparison of the average essential amino acid composition for both maize and sorghum seeds. Foods rich in lysine include legumes, dairy products and meat products such as fish and red meat. L-lysine is a crucial building block for proteins in the body. It also plays a major role in calcium absorption; muscle protein building; damaged tissue recovery; and proper production of the body's antibodies, hormones and enzymes. It is also believed that lysine plays a healing role in herpes simplex viral infections. The alleviation of cold-sores is lysine controlled as a speedy healing process is observed after lysine supply (Griffith *et al*., 1978).

Table 1.5 Comparison of the essential amino acid contents of sorghum and maize whole grain (mg/g crude protein)*.

Amino acid	Maize	Sorghum
Lysine	33.9	25.2
Histidine	30.4	21.4
Threonine	45.7	42.7
Valine	59.7	56.3
Isoleucine	50.4	56.3
Leucine	142.8	132.0
Methionine + Cysteine	48.6	50.1
Phenylalanine + Tyrosine	98.4	67.0

* = values based on crude protein contents of 8.5 and 10.3% for maize and sorghum respectively (Scherz and Senser, 1989)

Lysine bio-synthesis branch point depends on two feedback regulated enzymes called the aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS). In 1999, Mazur's group (Mazur *et al*., 1999) achieved an increment of total lysine content of up to twofold, this after expressing a feedback-insensitive DHPS in both the aleurone layer and the embryos of the maize seed. However, Mazur's group was unsuccessful in improving the lysine content in the starchy endosperm.

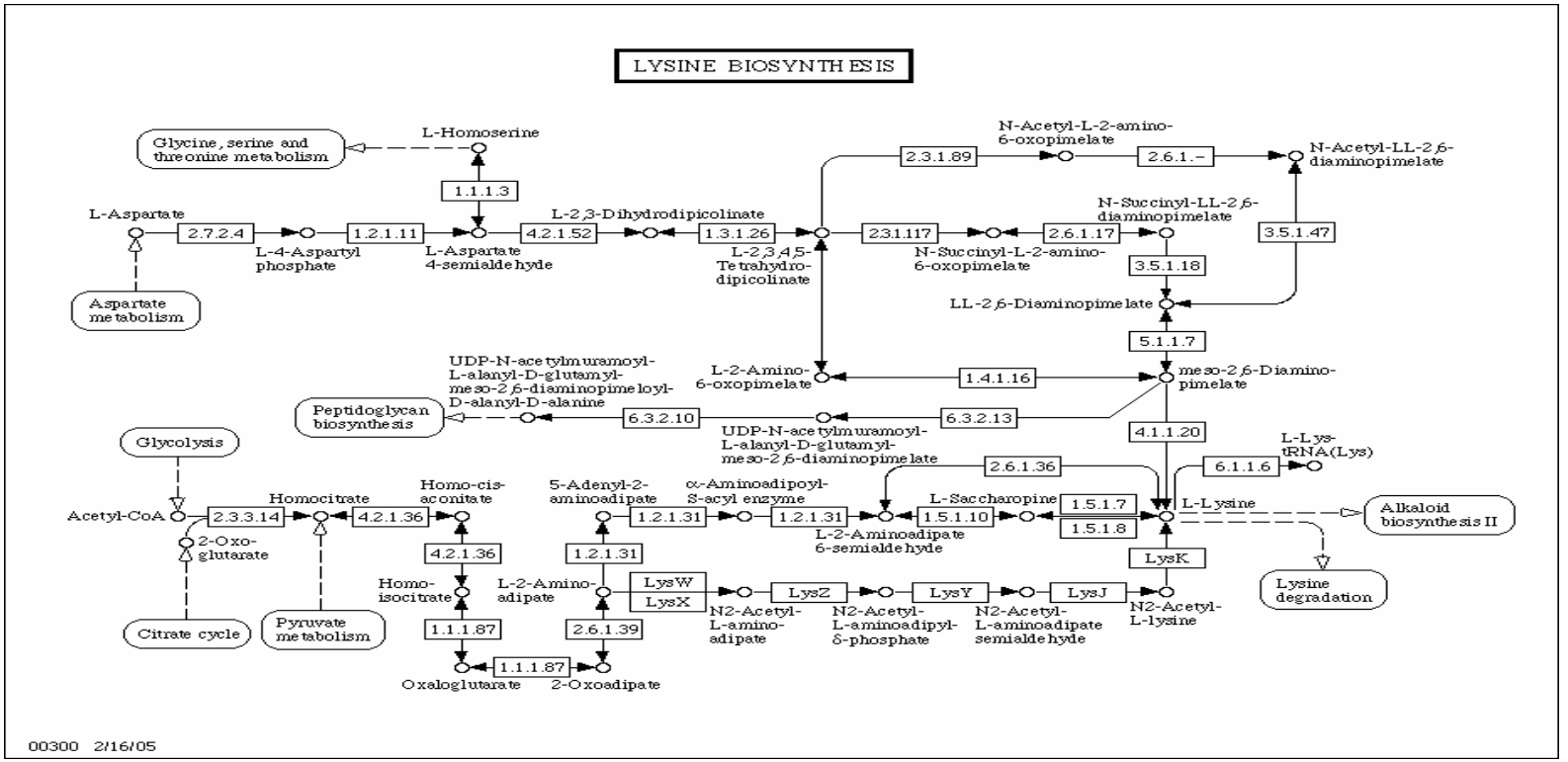

Figure 1.3 The lysine biosynthesis pathway. Numbered rectangular boxes represent substrate and product molecules (www.genome.ad.jp/kegg/pathway/map/map00300.html).

1.5.3 Seed protein digestibility

Protein bio-digestibility is a measure of how easy proteolytic enzymes catabolize a protein of interest. Proteins with superior digestibility have better nutritional quality because they yield more amino acids for absorption by the digestive system. Duodu (PhD thesis, 2000) rightly made mention of the fact that reported *in vitro* protein digestibility of uncooked sorghum and maize does not differ significantly. However, sorghum seed protein bio-digestibility significantly drops upon wet cooking in comparison with other cereals (Axtell *et al*., 1981; Hamaker *et al*., 1986; Hamaker *et al*., 1987). Duodu also suggested that wet cooking reduces bio-digestibility in sorghum via a re-conformation of α-helical structure of prolamins which could be a result of disulphide cross-links to form anti-parallel β-sheet formations. This was particularly observed with the high cysteine containing β- and γ-kafirins (Oria *et al*., 1995) suggesting more disulphide bond formations.

A few factors believed to be responsible for protein bio-digestibility include the following:

- the relationship between seed proteins and other seed components can reduce seed protein digestibility,
- phenolic compounds, especially tannins, can bind to proteins and render them less digestible,
- phytic acid chelates protein and minerals such as zinc and iron, thereby reducing the protein digestibility, and
- protein-to-protein cross-linkage assisted by wet cooking to form disulphide bonds.

1.6 Strategies for improving plant nutritional value: advantages of RNAi

Generally, initial efforts to improve plant nutritional quality were mainly directed at plant breeding. In the last two decades, genetic engineering for transgenic plant production has been an approach of choice (see section 1.4). Among other advantages for genetic engineering in plants is a wider scope and types of mutations that can be introduced. This is further strengthened by the ability to control the spatial and timing of transgene expression. This is vitally important as the plant's energy is conserved for other organs' metabolic functions and minimal disruptions are afforded to enable proper cultivation of the transgenic plant. In contrast, a lack of this spatial and timed manipulation is likely to possess deleterious effects. An example of this was the efforts to improve lysine content in plants by introducing a genetic mutation in the enzyme DHPS. It was deduced that targeted expression of transgenic DHPS in seeds as a result of driving expression by a seed-specific promoter avoids undesirable and deleterious effects in vegetative organs (Mazur, 1999).

RNA interference (RNAi) has been used in plants to improve the nutritional quality by targeted suppression of enzymes and other proteins that lead to the accumulation of desirable metabolites. Figure 1.4 shows a schematic diagram depicting the mechanism of RNAi gene silencing.

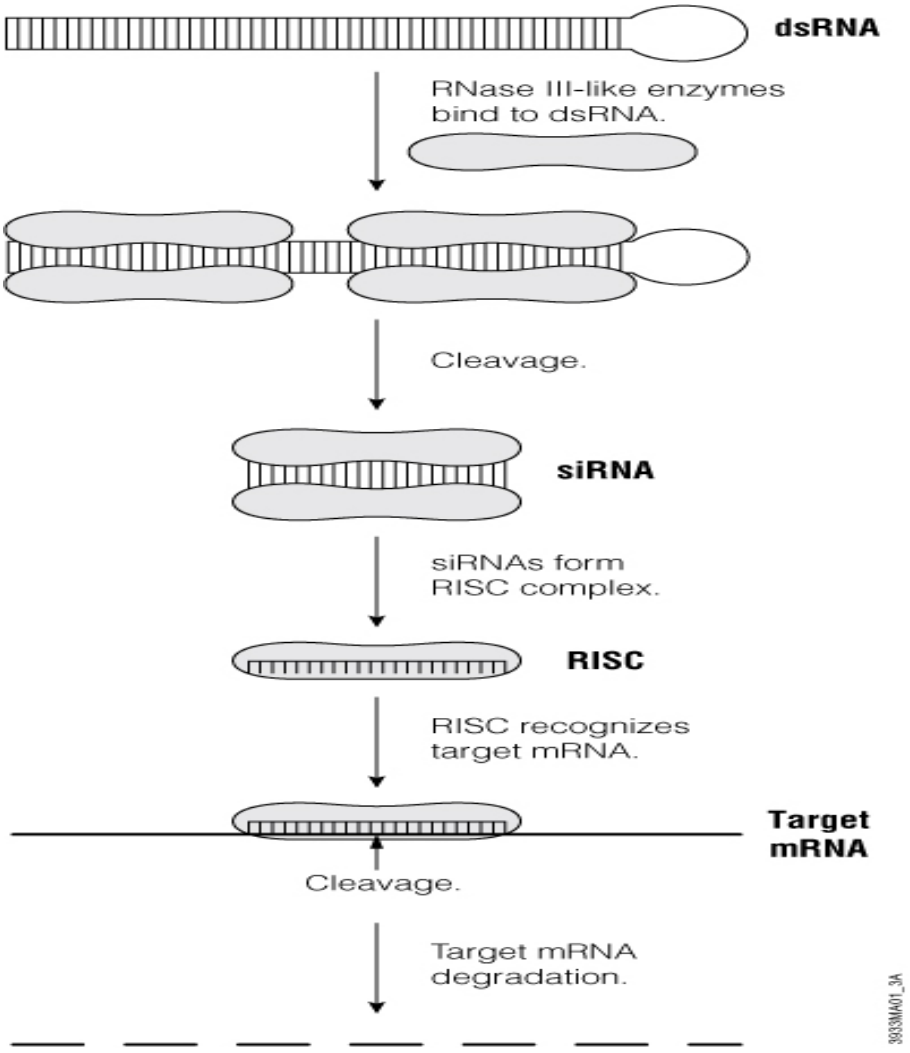

Figure 1.4 The principle of RNAi silencing. The small RNA molecules, microRNA (miRNA) and small interfering RNA (siRNA) bind to target RNAs and decrease their activity by preventing a messenger RNA from producing a protein. This process is instigated by the enzyme Dicer, which cuts long double-stranded RNA molecules into short fragments of ~20 nucleotides. This is called RNA-induced silencing complex (RISC). Photo taken from Promega website: http://www.promega.com/paguide/chap2.htm.

Using RNAi, lysine has been improved by targeting the suppression of the enzyme/s that effect lysine catabolism in seeds (Mazur *et al.*, 1999; Falco *et al.*, 1995). The RNAi technology has

also been used to reduce caffeine content in coffee plants by targeted suppression of the caffeine synthase gene (Ogita *et al*., 2003). A lysine-poor 22kDa alpha zein storage protein was successfully knocked out to improve the lysine content (Segal *et al*., 2003). In cotton, RNAi was used to improve the fatty acid quality content by co-suppression of two desaturase genes for the targeted improvements in the essential oleic and stearic fatty acids (Liu *et al*., 2002).

1.7 The rationale of the study

1.7.1 Problem Statement

Cereal seeds are generally poor in lysine and methionine and sorghum seeds, in particular, are very poor in the essential amino acids lysine and methionine contents as shown in Tables 1.3 and 1.5 (Scherz and Senser, 1989; Taylor and Belton, 2002). This deficiency in essential amino acid content is one of the two major determinants of sorghum's poor nutritional value, the other one being the low protein digestibility on wet cooking. The amino acid lysine is a first limiting essential amino acid for mammals in the sorghum seed. The endosperm accounts for most of the seed (80%) and it is the main storage compartment for seed storage proteins. Because prolamins comprise most of the endosperm proteins (Taylor and Schüssler, 1986), most of the attempts to improve the essential amino acid contents of cereals are directed at the prolamin fraction of seed proteins.

The major prolamins are α-kafirins, which comprise 70% of total prolamins. The α-kafirins are major contributors of the low lysine and methionine contents of the grain (Shewry and Halford, 2002). The second most abundant kafirin proteins are the γ-kafirins that appear on the outside of the protein body. These kafirins also contain very few lysine and methionine residues, and

therefore, are the second most contributors to the low seed lysine content. However, due to their hydrophobicity, their peripheral location on the protein body structure and their cysteine-rich nature resulting in increased di-sulphide bonds formation, in this study the γ-kafirins are identified as the major contributors to low seed lysine.

There are a few reports on attempts to enhance essential amino acid contents of cereal seed. These attempts include the suppression of the lysine catabolism enzyme LKR (Houmard *et al*., 2007) via the RNAi strategy in maize. Another approach reported involves the expression of an exogenous or heterologous protein that is rich in the amino acid of interest. One example was the expression of a γ-zein protein in various organs of the tobacco plant (Coleman *et al*., 1996). However, expression levels were very low. Technically, using this approach, it is imperative to produce single copy integration transgenic plants to effectively express the transgenic protein. There is also more demand on the seed protein resources due to the added protein expression required.

Yet another approach involved the manipulation of the lysine metabolic pathway to provide an increased source of the target amino acid, as was the case with the expression of a bacterial feedback-insensitive dihydrodipicolinate synthase (DHPS) in potato, tobacco, canola, soybean, barley, *Arabidopsis* and maize (Perl *et al*. 1992; Shaul and Galili, 1993; Falco *et al*. 1995; Brinch-Pedersen *et al*., 1996; Zhu and Galili, 2003; Huang *et al*., 2005). More recently, this approach revealed that the introduction of changes in the aspartate biosynthesis pathway often leads to changes in expression of non-targeted amino acids, especially those amino acids that are derived from the same precursor (Thu *et al*., 2007).

This study therefore attempts to address the sorghum grain's low lysine content, by using the RNAi approach to suppress two γ-kafirins, one δ-kafirin and the endosperm LKR protein suppression as a new and combined approach to improve the lysine content in sorghum. Because this approach is aimed at suppressing endogenous protein, the seed protein expression process will potentially be relieved for the expression of more valuable proteins. The LKR gene silencing aspect will ensure reduced lysine catabolic activity of the intended lysine increase.

Technically, sorghum has been classified as a recalcitrant cereal with regards to tissue culture regeneration and transformation (Kresovich *et al*., 1987; Zhu *et al*., 1998). For this reason, very few laboratories have reported successful sorghum tissue culture and transformation based manipulations in comparison to other cereal crops (Masteller and Holden, 1970; Cai *et al*., 1987; Ma and Liang, 1987; Cai and Butler, 1990; Casas *et al*., 1993, 1997; Kaeppler and Pederson, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000, 2003; Able *et al*., 2001; Emani *et al*., 2002; Tadesse *et al*., 2003; Gao *et al*., 2005; Howe *et al*., 2006). This presents a technical barrier to the use of genetic modification as a method of improving sorghum. This study addresses this barrier through improvement of the biolistics method of transformation.

1.7.2 Hypothesis

The application of RNAi technology to down-regulate selected seed lysine deficient endosperm kafirins, will result in the improvement in the nutritional quality of sorghum grain by increasing the seed lysine content. I postulate that removal of the select kafirins will avail amino acids for the accumulation of other, more beneficial, and therefore, an overall lysine content improvement.

1.7.3 Aims and objectives

This study aims to improve the seed lysine content by targeted suppression of kafirins that confer poor grain lysine content. Towards achieving the aim, improvements in the transformation process will have a major impact towards improving the sorghum plant. To make a meaningful research impact, the transformation efficiency and the ease of recovering stable transgenic plants were targeted to increase the sorghum manipulation effort *in vitro*. Four of the five sorghum genotypes that were chosen for the transformation experimentation were never tested for tissue culture amenability before.

To achieve the aims stated above, the following objectives were set as follows:

- evaluation of tissue culture amenability of five sorghum genotypes,
- sorghum biolistic transformation protocol optimization and generation of independent transgenic events with a selected vector
- characterization of transgenic plants for kafirins suppression and seed lysine improvements.

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Chapter 2

Screening of sorghum genotypes for tissue culture amenability

2.1 Abstract

Sorghum genotype responsiveness to *in vitro* culture and manipulation is essential to establish a routine transformation protocol. This entails identification of those genotypes that display desirable tissue culture response, in particular somatic embryogenesis coupled with whole plant regeneration in a defined tissue culture medium. The regeneration capacity of five sorghum genotypes on three different solid nutrient media was investigated, i.e. new genotype X medium combination. The most responsive genotypes were identified based on their ability to form totipotent callus cultures that give rise to plantlets with normal shoots and root systems within the shortest culture period *in vitro*. It was found that regeneration of plants was mainly through somatic embryogenesis via type I callus. The genotype P898012 on culture medium previously designated medium J gave the best callus induction of 98%, the highest regeneration potential of 6.13 regenerants/explants and the shortest tissue culturing period of 74 days. This genotype and medium combination has been therefore selected for subsequent transformation and regeneration of sorghum transgenics with improved traits.

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2.2 Introduction

Somatic embryogenesis is defined as a process in which somatic cells undergo bipolar development resulting in whole plants through the development of globular structured embryos. These embryos differentiate into plants that are genetically identical to their parental somatic embryo cells. The resulting plants display growth patterns that are similar to that of seed-derived plants.

High frequency plant regeneration from cultured explant material is a prerequisite for successful transformation of most cereal crops. One of the key limiting steps in the development of genetic engineering protocols for the improvement of cereal crops through biolistic and *Agrobacterium*-mediated transformation is the *in vitro* plant regeneration process. *In vitro* culture of cereals shows strong genotype dependence and production of the appropriate culture is generally limited to selected genotypes. There is also strong evidence to suggest that sorghum is no exception to this genetic control (Tomes and Smith, 1985; Morocz *et al*., 1990).

This part of the study addressed the problem of callus initiation and regeneration potential of five sorghum genotypes that originate from major sorghum growing areas in Africa, cultured on specific nutrient media formulations. The objective was to identify the best genotype-nutrient medium combination that results in satisfactory regenerability for subsequent transformation activities.

2.3 Materials and methods

2.3.1 Sorghum genotypes and explant

Five sorghum [*Sorghum bicolor* **(L.)** Moench] genotypes chosen for tissue culture amenability screening were Kapaala (Indian), Kadaga (Ghanaian), SA 2861, SA 4322 (both South African genotypes) and P898012 (American). The genotypes were chosen on the basis of agronomic and functional attributes ranging from good food and brewing qualities (Kapaala and Kadaga) to stem borer and aphid resistance (SA 2861 and SA 4322, respectively) and transformability (P898012).

2.3.2 Plant material and nutrient media

Immature zygotic embryos (IZEs) ranging from 0.8-1.2 mm in length were used as explants. These were derived from sorghum seeds harvested 12-15 days post anthesis. The immature seeds were surface-sterilized in 70% (v/v) ethanol for 3 min, and for 15 min in 2.5% sodium hypochlorite solution containing 0.1% Tween-20 before a thorough rinse with sterile distilled water. Tissue culture experiments were performed under aseptic conditions. Callus cultures were transferred to fresh callus induction medium (CIM) every two weeks until the onset of somatic embryogenesis. The IZEs were placed on CIM with the scutellum cells facing upwards and the embryogenic axis in contact with the CIM for somatic embryo formation. After somatic embryo formation, the calli were transferred to callus maintenance medium (maturation medium) before transfer to regeneration medium for plantlet production. In total, 100 to a 150 IZEs (10 embryos per petri dish) were cultured per genotype per tissue culture medium (Table 2.4). The IZE explants consisted of three biological replicates, i.e. independent panicles and harvest dates, with 15-30 explants per replication.

Tables 2.1 to 2.3 list a summary of the nutrient media contents and the culturing conditions for each solid medium used in this study. For tissue culturing on medium J (O'Kennedy *et al*., 2004), the CIM contains L3 based salts and vitamins (see Annexure), 2.5 mg l^{-1} of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), the carbon source maltose, 4 g/l gelrite as the gelling agent and 20mM L-proline. In the root and regeneration medium (RRM), 2,4-D and L-proline were not included. A 4-week culture on CIM was followed by a two-week period on callus maturation medium, prior to regeneration and rooting regimes/phases. The maturation medium contained double the amount of carbohydrate, which in this case was maltose (Table 2.1).

For tissue culturing on Tadesse's medium, somatic embryogenic calli formed within 4 weeks of culture on CIM were transferred to a modified CIM with reduction in 2,4-D (2.5 – 2.0mg/l) and increment in kinetin (0.2 – 0.5 mg/l) until somatic embryos were ready to germinate (Table 2.2). The somatic embryos were then transferred to shoot induction medium (SIM) until shoots developed (Tadesse *et al*., 2003) and subsequently to root induction medium (RIM).

The CAPD medium was described by Casas *et al.* (1993). This medium was used with the following modifications: 1 g/l asparagine and 2 mg/l 2,4-D was added in the CIM. After 14 days on CAPD2 and 7 days on CAPD1 to initiate somatic embryoids, the cultures were transferred to callus maintenance medium (CCM) for 4-7 days, followed by subsequent culture for 2-6 weeks on regeneration medium. The callus regenerating medium (CMR) was responsible for shoot formation within 14-28 days. The callus shoot elongation (CSE) medium cultures take 10-14 days before shoots are cultured on Casas Rooting Medium (CROOT) for 14 days. Finally,

transfer and culturing of rooted shoots on Casas Root Elongation (CRE) for 7-14 days was performed (Table 2.3).

2.3.3 Data collection and statistical analysis

The incubation conditions for all cultures were at 24-25˚C under low-light conditions (1.8 $\mu Em^{-2}s^{-1}$) except for regeneration of shoots (≥ 5cm), which were incubated under light conditions of 18 $\mu Em^{-2}s^{-1}$. The rooted plantlets with a height of 5-10 cm were considered as 'regenerants'. A random selection of regenerants was hardened off to be assessed for fertility in the greenhouse. Data on percentage callusing and regeneration ratio was entered into Excel and statistical analysis was carried out using the MINITAB software Release version 12.21 (MINITAB Inc., 1998). The experiment was a two-factor factorial experiment (genotype and media) in a Completely Randomized Design with replication where the genotype and media are the factors. The Tukey's Test was used to perform a pair-wise comparison of the means of the genotypes.

Table 2.1 Composition of tissue culture medium J.

Nutrient	**J CIM**	**J Maturation medium**	**Medium J RRM**
L3 macro- and micro elements	+*	+*	+*
MS-Fe source	+*	+*	+*
HL2 Vitamins	+*	+*	+*
2,4-D	2.5mg/l	-	-
Maltose	30g/l	60g/l	30g/l
pH	5.8	5.8	5.8
Gelrite	4g/l	4g/l	4g/l
L-Proline	20mM	-	-

* = see Annexure

Table 2.2 Composition of Tadesse's tissue culture medium.

Nutrient	**Tadesse's CIM**	**Tadesse's modified CIM**	**Tadesse's shoot induction medium**	**Tadesse's root induction medium**
MS macro- and micro elements	+*	+*	+*	½ strength
MS-Fe source	+*	+*	+*	½ strength
Jacobs Vitamins	+*	+*	+*	+*
2,4-D	2.5mg/l	2mg/l	-	-
Kinetin	0.2mg/l	0.5mg/l	0.5mg/l	-
IBA	-	-	-	0.5mg/l
NAA	-	-	-	0.5mg/l
Sucrose	30g/l	30g/l	30g/l	20g/l
pH	5.8	5.8	5.8	5.8
Agar	8g/l	8g/l	8g/l	8g/l

* = see Annexure

Table 2.3 Composition of CAPD2 tissue culture medium.

Nutrient	CAPD2	CAPD1	CCM	CMR	CSE	CRoot	CRE
MS Macro-, micro- & Fe source	+*	+*	+*	+*	+*	½ strength	½ strength
B5 modified Vitamin (Ca-pantothenate)	+*	+*	+*	+*	+*	+*	+*
2,4-D	2mg/l	1mg/l	1mg/l	-	-	-	-
Kinetin	-	-	0.5mg/l	0.5mg/l	0.5mg/l	-	-
IAA	-	-	-	1mg/l	1mg/l		
IBA	-	-	-	-	-	0.5mg/l	-
NAA	-	-	-	-	-	0.5mg/l	-
NH_4NO_3	3.3g/l	3.3g/l	-	-	-	-	-
Proline	2g/l	2g/l	-	-	-	-	-
Asparagine	1g/l	1g/l	-	-	-	-	-
$AgNO_3$	-	-	-	10mg/l	-	-	-
Coconut water	100ml/l	100ml/l	-	-	-	-	-
Sucrose	30g/l	30g/l	30g/l	30g/l	30g/l	20g/l	20g/l
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Agar	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l

* = see Annexure

2.4 Results

Sorghum IZEs proliferated to produce two types of calli, a very soft and watery non-embryogenic callus (Figure 2.1 A), and a highly embryogenic totipotent type I callus that is harder and white in appearance (Figure 2.1 B). Table 2.4 shows the regeneration data for the genotypes under different media conditions. Using analysis of variance (ANOVA) for nine replicated experiments, data showed that the response to both callus induction and regeneration were influenced independently by medium and genotype. Thus their interaction had a stronger effect (probability values in Table 2.5, last column) where P values below 0.05 indicate significant differences. Overall, pair-wise comparison ranked the genotypes in the order of P898012>SA2861>Kadaga>SA4322 for callus induction on the three media, with genotype P898012 producing 98% somatic embryos on medium J.

The coefficient of variation for the callus induction and regeneration (18.8% and 6.1%) suggested that the experiments were reproducible with low experimental error. The coefficient of variation value above 40% points to low reproducibility of the data. This value is indicative of a variation of data points in comparison to the mean value. Therefore the values attained in this part of the study, i.e. below 20%, indicate that the data is reliable. A dissection of the influence of media on the genotypes also revealed a significant medium formulation effect; this was displayed by the probability values (Table 2.5 A and B; last column). Again, the P-values below 0.05 suggest a significant effect of the medium composition on both somatogenesis and regeneration. A probability value above 0.05 suggests a strong probability of no significant difference caused by the parameter tested. The significant effect caused by the genotype was further indicated by the results for Kadaga. Fifty percent of Kadaga IZEs proliferated and formed

somatic embryos on medium J, while genotype SA 4322 IZEs did not proliferate on the same medium J.

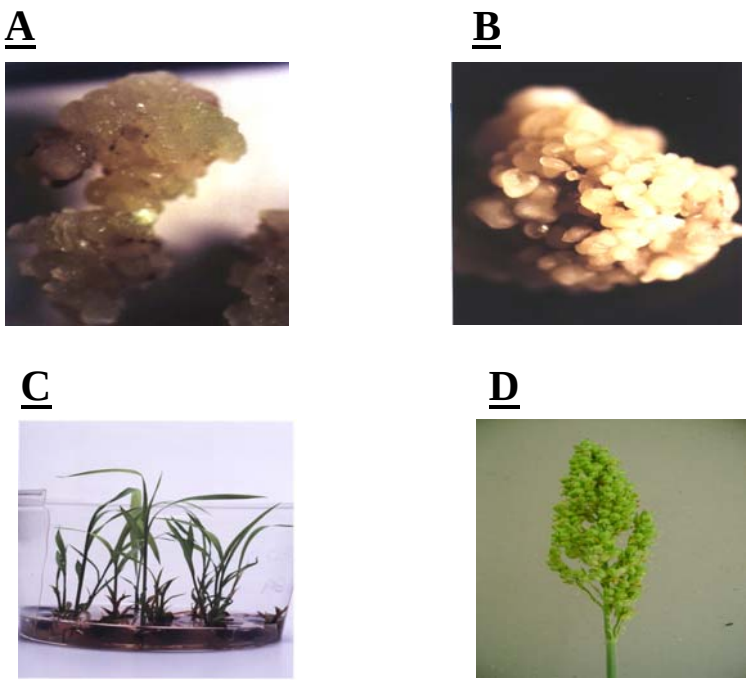
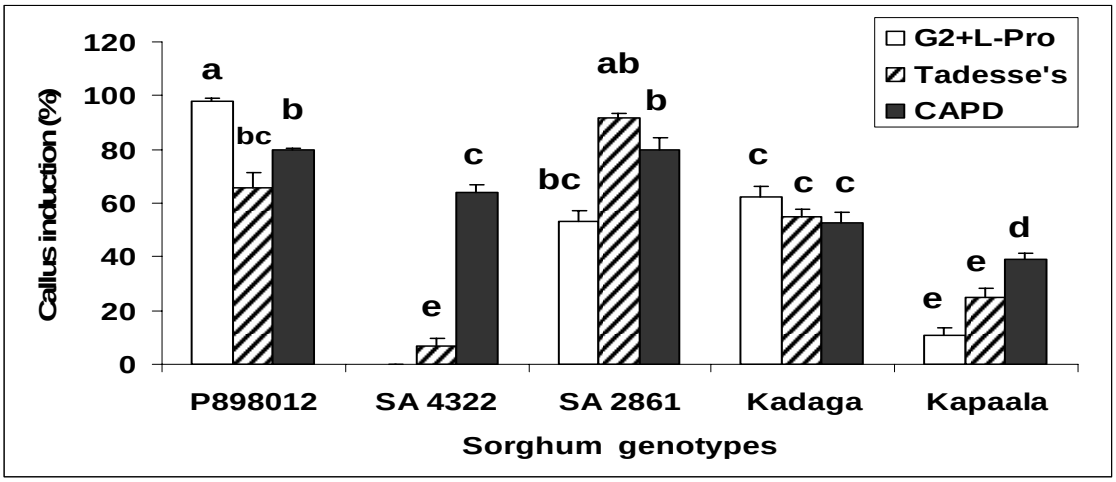


Figure 2.1 *In vitro* plant regeneration from immature zygotic embryos of sorghum. (A) Non-regenerable, soft and watery callus produced/formed by some embryos. (B) White, compact embryogenic type I tissue derived from cultured IZEs of genotype P898012 on tissue culture medium J within two weeks. (C) Sorghum genotype P898012 plantlets shooting and rooting on regime J regeneration medium. These plantlets resulted in fertile F_0 plants. (D) Mature sorghum head from tissue culture plants.

The regeneration potential of the five genotypes was calculated as the number of regenerants divided by the number of explants cultured and results are shown in Figure 2.2 and Table 2.4. Overall, the genotype responsiveness was ranked in the order of P898012>SA 2861>SA4322>Kapaala>Kadaga in all three media formulations tested. On medium J, P898012 was found to be the most regenerable genotype at 6.13 regenerants/explants (reg./expl.), followed by genotype SA 2861 at 1.01 reg./expl. On Tadesse's medium, P898012 was also found to be superior in comparison to the other four genotypes, yielding 1.4 reg./expl. On CAPD medium, the two South African genotypes were the best performers, yielding 3.56 reg./expl. for SA 4322 and 1.71 reg./expl. for SA 2861. Kapaala showed some regenerability (between 0.01 and 0.17 reg./expl.) while no regeneration was obtained for Kadaga in all three media formulations tested. Visual screening revealed that all F_0 sorghum genotypes that were hardened-off showed phenotypes with unchanged vegetative growth and viable F_1 seeds after self fertilization. Another observation was that genotype P898012 on medium J seemed to produce plantlets much faster (75-90 days) when compared to the other two (100-140 days).

A



B

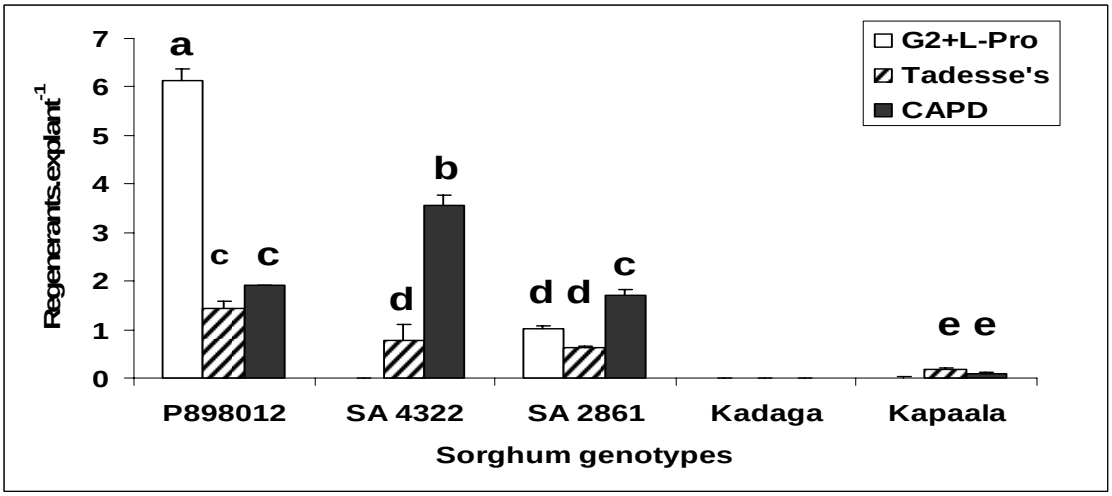


Figure 2.2 The effect of culture media on callus induction (A) and plant regeneration (B) of five sorghum genotypes from immature zygotic embryos. The bars represent the mean of nine individual experiments with ±SE (standard error). The most totipotent calli were produced by P898012 on J medium which resulted in 6.13 regenerants per explant. Bars with the same letter are not significantly different (P>0.05).

Table 2.4 Results of plant regeneration observed for each of the genotypes on the three culture media tested.

Genotype	Medium	Number of IZEs	Number of somatic embryos produced	Number of reg./expl. (expl. calculated from number of somatic embryos formed)	Days on tissue culture
P898012	J	146	143	6.13	74-100
SA 4322	CAPD	135	86	3.56	100-140
SA 2861	CAPD	134	107	1.71	100-140
P898012	Tadesse's	113	70	1.4	100-144
SA2861	J	150	80	1.01	120-140
SA4322	Tadesse's	135	9	0.77	100-144
SA2861	Tadesse's	132	121	0.63	120-144
Kapaala	Tadesse's	147	37	0.17	120-140
Kapaala	CAPD	100	39	0.10	120-140
Kapaala	J	147	16	0.02	120-140

Table 2.5 ANOVA for regeneration statistics (**A**) callus induction in sorghum genotypes, and (B) regeneration in sorghum genotypes. Nine individual experiments were carried out. * shows statistical significance at 5% level.

A

Source	Degrees of Freedom	Mean Square	*F* value	Probability
Replication	9	69.9	0.72	0.686
Genotype	4	20153.8	208.53	0.000*
Media	2	4141.6	42.85	0.000*
Genotype x Media	8	3750.9	38.81	0.000*
Error	113	96.6		

Coefficient of variation: 18.8%

B

Source	Degrees of Freedom	Mean Square	*F* value	Probability
Replication	9	0.148	0.84	0.582
Genotype	4	44.169	250.32	0.000*
Media	2	10.274	58.23	0.000*
Genotype x Media	8	22.315	126.47	0.000*
Error mean square	113	0.176		

Coefficient of variation: 6.1 %

2.5 Discussion

This study tested the response of five genotypes, of which two were from South Africa, cultured on three different media. The sorghum genotypes displayed different responses to *in vitro* culture where P898012 showed the highest regeneration capacity on medium J, supplemented with L-proline. These varied responses show clear interactions of the genotypes with the different nutrient media. Observed genotype-medium interactions are consistent with what has been observed for other cereals (Khanna and Raina, 1998). This has resulted in the identification of model genotypes for transformation purposes in other cereals, such as genotypes T309 and IR72 in rice (Christou, 1994), the A188 in maize (Ishida *et al*., 1996; Locatelli *et al*., 1992), Golden Promise in barley (Jacobsen *et al*., 1999; Tingay *et al*., 1997), and Bobwhite genotypes in wheat (Altpeter and Varshney, 2001; Wu *et al*., 2003).

In this study, P898012 on medium J gave an embryogenic callus percentage of 97.9%, the highest reported for sorghum IZE as explants. This embryogenesis potential value indicates a further improvement to what had already been reported, i.e. 81% for genotype P898012 cultured on different tissue culture media containing naphthalenacetic acid and an increased nitrogen and potassium phosphate contents (Sato *et al*., 2004).

This report also states a specific regeneration potential of 6.13 reg./expl. value for P898012 on medium J. Most sorghum regeneration reports do not provide such reg./expl. values. Instead, they report on somatic embryo callus formation potential as a measure of tissue culture amenability (Tadesse *et al*., 2003; Casas *et al*., 1993). This reg./expl. value is, however,

important for *in vitro* regeneration of the sorghum IZEs, since the callus formation potential is not always directly related to the regeneration potential. This is due to medium composition used for callus formation and regeneration purposes. Both medium compositions are different processes involving different medium formulations. This was reported first by Khanna and Raina (1998) while investigating the genotype X nutrient media interactions effects on regeneration in three indica rice cultivars. They concluded that regeneration percentage was influenced by the genotype, callus induction medium, regeneration medium and interaction between these parameters. The present study extends this knowledge since the genotype SA2861 X CAPD medium gave a 79.9% callus formation rate. This was inferior to the 91.7% rate achieved by the genotype SA2861 on Tadesse's medium. Furthermore, the former combination also had a superior reg./expl. value of 1.71 in comparison to the 0.63 value for the latter combination. The regeneration process should, therefore, be treated independently where the regeneration medium can be altered independent of the CIM.

In this study, the regeneration capability was measured in addition to the callus formation potential of five sorghum genotypes on three media formulations. This extends the knowledge on sorghum tissue culture performance as a first step to genetically engineer this cereal crop. The different callus responses and subsequent plant regeneration potential of different genotypes on identical nutrient medium is likely due to the genetic (genotype) X environmental (medium) interaction as stated by Khanna and Raina (1998). Also, the same environmental conditions (medium) can result in different callus responses and this could be a result of different genetic control factors (Tomes and Smith, 1985; Morocz *et al*., 1990). Other factors, such as the size and physiological state of the explants that could affect callus totipotency were eliminated by

experimental design in this study. All explants were carefully chosen to exclude explant sizes that could either be too small or too big. The best embryo size was chosen as 0.9-1.2mm long. Each seed batch of a particular genotype was equally divided by culturing on all three nutrient media. This ensured that a uniform callus response from explant to explant and medium plate to medium plate was achieved (Table 2.5).

The period of *in vitro* culturing, indicated by days to regeneration, is also a useful indicator for rapid tissue proliferation because it is a measure of how fast the callus tissue divides to reach the regeneration stage. The most regenerable line also produced the shortest *in vitro* culturing period, i.e. earliest plantlets produced after 74 days with P898012 on medium J. This is especially crucial in the production of healthy transgenic plants as the stress imposed by tissue culture is minimized, and so is the opportunity for somaclonal variation due to lengthy tissue culture conditions. Somaclonal variation is defined as the phenotypic variation that is often observed in plants as a result of the tissue culture stress and is suspected to be caused by chromosomal re-arrangements.

In conclusion, the sorghum genotypes tested showed a variation of genotypic responses to *in vitro* culture. This further extends our knowledge that was previously reported in indica rice that the callus formation and regeneration potential is dependent on the genotype and medium composition. From the results in this study, the chosen sorghum genotypes for transformation experiments were, in order of priority, P898012 on medium J, followed by SA 4322 and SA 2861 on CAPD. These combinations displayed superior sorghum regeneration potential and it is anticipated that it should significantly increase the probability of producing transgenic sorghum

plants. In the following chapter, the optimization of the transformation selection system and sorghum transformation was carried out by using genotype P898012 on medium J.

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