CHARACTERISATION OF *AMARANTHUS TRICOLOR* MUTANT PLANTS WITH INCREASED DROUGHT-TOLERANCE

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ABSTRACT

Amaranthus tricolor (*A. tricolor*) is a nutritious vegetable crop that is used as a subsistence and cash crop in the rural areas in Africa. Its yield and production is severely limited by abiotic stresses such as drought. Mutation technology, using gamma irradiation, was previously employed as a tool to create genetic variation in order to select for lines with improved drought-tolerance. During irradiation, 160 Gy (Gray) was selected as the optimal dosimetry that allowed subsequent seed germination. The resulting mutant lines were screened over several generations under field and greenhouse conditions and seven promising drought-tolerant lines were selected. Here we report on physiological and morphological studies of two of these *Amaranthus* mutant lines (#2 and #5) to confirm the enhanced drought-tolerance. Plants were grown in the greenhouse in plastic pots containing germination mix with fertiliser. They were exposed to 21 days of well-watered conditions, 19 days of drought-stress conditions and 7 days of re-watering. Shoot height, leaf area, protein content and relative water content (RWC) of the fresh and dry material were determined colorimetrically under well-watered and drought-stress conditions, while anthocyanin was only measured during well-watered conditions. Shoot height, leaf area, number of leaves per plant and the protein content were significantly reduced under water-stress conditions. Under well-watered conditions, mutant #5 grew faster with the shoot length been significantly higher than mutant #2 and the wild type. Even though drought adversely affected shoot length, mutant #5 still performed better than mutant #2 and the wild type under drought-stress conditions. While under both well-watered and drought-stress conditions, the wild type plants had bigger leaf area compared to the two mutant lines. After 16 days of drought-stress conditions, all the leaves of the wild type plants were dried out, as a result no wild type plants recovered after 8 days re-watering. Meanwhile, both mutant #2 and mutant #5 plants recovered significantly after 8 days of re-watering. The wild type was affected more by drought-stress, therefore the wild type was classified as less drought-tolerant compared to the two mutant lines. Protein content for mutant #2 plants was higher under both well-watered and drought-stress conditions but was not significantly

different from mutant #5 plants compared to the wild type plants after 19 days of drought-stress conditions. RWC decreased in all the *Amaranthus* lines (wild type, #2 and #5) after 19 days of drought-stress conditions. Furthermore, genetic diversity was examined in all the *Amaranthus* lines using random amplified polymorphic DNA (RAPD) analysis. Nineteen arbitrary RAPD markers were used of which two detected polymorphisms (OPA 07 and OPA 16).

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ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	Degree Celsius
ABA	Abscisic acid
AFLP	Amplified Fragment Length Polymorphism
BSA	Bovine Serum Albumin
C_4	Carbon 4
cDNA	complementary DNA
cm	Centimeter
dH_2O	Sterile distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DW	Dry Weight
FW	Fresh Weight
µg	Microgram
g	Gram
hr	Hour
Gy	Gray
H_2O	Water
HCl	Hydrochloric acid
L	Liter
rpm	revolutions per minute
pH	Log Hydrogen ion concentration
µL	Microliter
µM	Micromolar
M	Molar
$MgCl_2$	Magnesium Chloride

Mg	Milligram
mL	Milliliter
min	Minute
mm	Millimeter
mM	Millimolar
MPa	MegaPascal
MS	Murashige and Skoog
N	Normality
nm	Nanometer
OD	Optical density
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
RAPD	Random Amplified Polymorphic DNA
RDA	Representational Difference Analysis
RWC	Relative Water Content
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
Tris	Tris(hydromethyl) aminomethane
TW	Turgor Weight
SA	South Africa
SE	Standard Error
UK	United Kingdom
UV-A	Ultraviolet A radiation
UV-B	Ultraviolet B radiation
V	Voltage

CHAPTER 1

1. Introduction

1.1. General Introduction

Amaranthus plants can be used as a pseudo cereal crop, as well as a leafy vegetable. Attention to this crop is increasing, because of its tolerance to drought-stress, high disease and pest resistance, and high yield in production even on poor soils (Becker *et al.*, 1981; Bressani *et al.*, 1987). It is also very palatable, easy to cook and has high nutritional values which are required for human diet especially in developing countries where food is expensive or scarce. These qualities have made this crop particularly interesting for developing countries (van den Heever *et al.*, 2007). Furthermore this crop also exhibits tremendous morphological diversity and extensive adaptation to various eco-geographical situations (Ray and Roy, 2009). Due to this plant's qualities and its ability to withstand harsh conditions, some believe that improvement of this crop can contribute to the world's food supplies, thus improving the economies of many countries. It could also lessen hunger in marginal areas while improving the income of the farmers (van den Heever *et al.*, 2007). Unfortunately this crop has received minimal attention in terms of research and many people don't know much about its overall nutritional quality. Improving drought-tolerance in this important crop will undoubtedly contribute to food security and enhance the quality of life for people who depend on it. For this reason, knowledge of the crop's nutritional values, its ability to acclimatise and to grow well under different weather and soil conditions is essential.

Modern plant breeding and biotechnological techniques can be used to further enhance traits such as drought-tolerance in these crops. Radiation can be used to change different characteristics of a plant, and finally improve certain traits of interest. In 1999, 48 000 *A. tricolor* seeds (syn. *Gangeticus*) were vacuum packed in plastic bags and sent to Atomic Energy Corporation, Pelindaba to be gamma radiated. Seeds were improved by inducing

mutations followed by selection for lines with improved traits such as drought-tolerance. Radiation is known to cause changes to the plant genome, but plant performance between plants may, however, be similar (Jie *et al.*, 1993). Hence, this study seeks to investigate the physiological responses of irradiated and wild type *Amaranthus* plants during drought conditions. This study will be conducted in a temperature-controlled environment in the greenhouse. Imposing controlled water-stress in the field is difficult because of unpredictable rainfall and soil heterogeneity making it difficult to interpret the results.

1.2. RESEARCH AIM

Abiotic stresses such as drought constitute a major constraint for agricultural production in many developing countries. Crops of agricultural importance that are already tolerant to drought-stress can significantly contribute to food security thus improving the economy of the countries. *A. tricolor* was improved at the Atomic Energy Corporation, Pelindaba by inducing mutations and as a result produce varieties with improved traits such as drought-tolerance. The aim of this study was to evaluate the physiological characteristics of these *A. tricolor* mutants with increased drought-tolerance in comparison to the wild type plants.

1.3. OBJECTIVES

The specific objectives of this study were to:

1. Establish the best growth condition for the *Amaranthus* plants.
2. Greenhouse trial in order to determine the physiologically and morphologically characterise drought-tolerant mutant plants under well-watered and drought-stressed conditions in comparison to the wild type plants.
3. Analyse the genetic diversity of genomic DNA isolated from *Amaranthus* plants using random amplified polymorphic DNA (RAPD).

CHAPTER 2

2. Literature Review

2.1. Plants and stress

Plants are bound to their habitats and not all the plants can survive under arid environmental conditions. Those that do, usually only survive for a limited period of drought-stress. Plants cannot remove themselves from any unfavourable environmental or anthropogenic stressors, therefore they require a special mechanism to adapt and avoid stress in order to survive. When plants are exposed to stressful conditions such as drought, the demand for water increases, leads to destabilisation of cellular functions and finally to permanent damage or even death. Any environmental factor likely to affect living organisms can be regarded as stressful (Levitt, 1972).

Plant responses to drought vary depending on the species and their genotype, the cell type and its subcellular compartments, the age and stage of development, the period of drought and the severity of water deficit (Bray, 1997). Therefore, different species in response to environment vary due to their differentiation in water use efficiency. Plants with high water efficiency are predicted to have an increase in biomass per unit water lost during drought conditions than those with less water use efficiency (Heschel *et al.*, 2002).

Plant stressors are divided into two factors; environmental (abiotic) and living (biotic) stresses. Abiotic stress factors include: drought, heat, high light, wind, storm, fire, mineral deficiency, salinity, low temperature, wounding, UV-A, UV-B, flooding and ozone. Biotic stress includes: insects, pathogens, bacteria, fungi, virus and higher animals including human interference (Figure 1). Abiotic stresses such as drought and salinity are the major cause of crop loss in many parts of the world (Katerji *et al.*, 2001). In most countries where food supply is insufficient for the growing population, plant

biologists are working on implementing modern techniques that will help in overcoming environmental stresses such as drought (Vinocur and Altman, 2005). An understanding of the physiological mechanisms and genetic controls of traits such as drought at various stages of plant development is essential in order to develop crop plants with improved traits like drought-tolerance (Ashraf and Foolad, 2007).

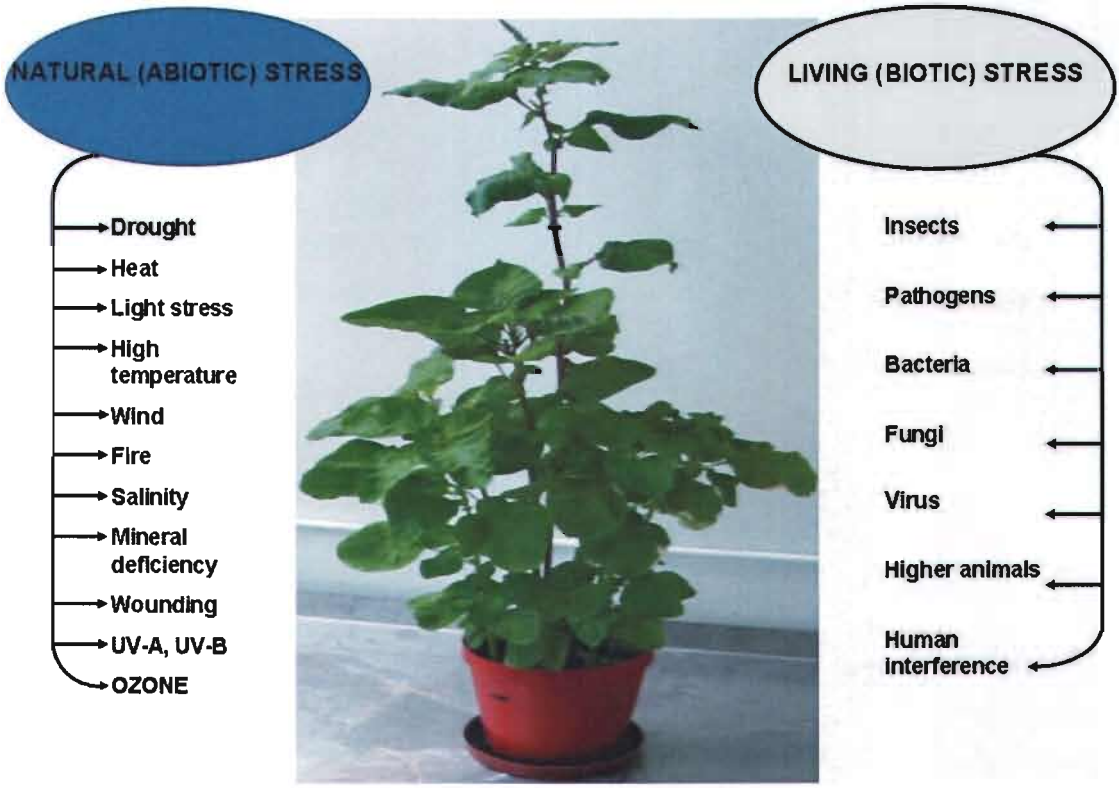

Figure 2: Abiotic (natural) and biotic (living) stress factors that affect plant growth

2.2. Drought-stress in plants

Drought-stress causes insufficient availability of water for plants, animals and human life. Crops of agricultural importance exhibiting high drought-tolerance, such as *Amaranthus*, can contribute to food security. However, the plants' quality under stressful

conditions can be decreased (O'Brien and Price, 1998) and may show alteration of morphological and physiological features, as well as biochemical and molecular processes (Lawlor and Cornic, 2002). The mechanisms involved during drought-stress are still a challenge because drought-tolerance is a complex trait which consists of several metabolic pathways. In order to increase the efficiency in developing drought-tolerant varieties, one needs to understand, identify and isolate genes associated with drought-tolerance. The mechanisms of drought-tolerance are triggered by the response of roots to water limitation since the roots are the primary site for stress signal perception in which a cascade of gene expression is initiated. These transcriptional changes can lead to plant adaptation to drought-tolerance by controlling gene expression and signal transduction in drought-stress response (regulatory proteins) or protecting the plant against environmental stress (functional proteins) (Périn *et al.*, 2007). Extending the plant's exposure to drought-stress forces the plant to adapt to stress conditions.

Fukai and Cooper (1995) reported that morphological mechanisms become relentlessly affected when the plants are exposed to a rapid water shortage. Liu and Stützel (2002) have previously reported the physiological adaptation of vegetable *Amaranthus* in response to drought-stress showing evidence of high osmotic adjustment (1.08-1.24 MPa) in all *Amaranthus* genotypes which guaranteed that the plants continue to progress well even under drought-stress conditions. Slabbert *et al.* (2004) reported that amaranth plants can withstand drought-stress by means of mechanisms of osmotic, metabolic and photosynthetic adjustment. *Amaranthus* is able to control transpiration (evaporation) water loss by closing the stomata (Cornic 2000; Lawlor and Cornic, 2002; Efeoğlu *et al.*, 2009). As a consequence, turgor-dependent processes such as growth and stomatal activity gradually decreases the leaf water potential (Liu and Stützel, 2002).

Drought related responses such as stomatal closure are mediated by abscisic acid (ABA). An increase in ABA can cause many changes in plant growth, development and its physiology. When the rate of transpiration is greater than the uptake of water, plant or

cellular water deficit occurs, and results in the reduction of RWC, cell turgor and cell volume (Lawlor and Cornic, 2002).

2.3. Drought Avoidance Mechanism

The strategies that plants can use to adapt to drought-stress conditions include drought avoidance, drought escape and drought-tolerance. This means that plants can either avoid drought by maintaining their water status in the presence of drought-stress, or escape drought by completing its life cycle before the beginning of a drought period, or plants can tolerate drought by functioning under drought-stress conditions (Levitt, 1980; Turner *et al.,* 2001). Plants can maintain the available water within the cell compartments during drought by avoiding dehydration (Valliyodan and Nguyen, 2006). Even though several studies have revealed that the presence of water plays an important role within plant growth, only a few studies have shown the adaptive genetic differentiation in response to variation in drought conditions (Heschel *et al.,* 2002) while some studies have previously reported the response of rice seedlings to drought-stress conditions (Cabuslay *et al.*, 2002; Reddy *et al.,* 2002; Salekdeh *et al.,* 2002; Rabbani *et al.,* 2003).

There are several characteristics that help the plant to survive under drought-stress conditions. These are achieved by modulation of gene expression and synthesis of osmoprotectants. Accumulation of molecules which protect specific cellular functions plays an important role in stabilisation of the cell membrane and can guarantee recovery of the plant when the soil is rehydrated (Munns, 1988; Liu and Stützel, 2002; Reddy *et al.,* 2004; Valliyodan and Nguyen, 2006; Zang and Komatsu, 2007). A process in which the solutes are accumulated in the plant tissue due to lower water potential allow the plant to retain its turgor and is called osmotic adjustment (Liu and Stützel, 2002; Nayyar and Walia, 2004).

More long-term adaptation mechanisms involve, for instance, changes in root to shoot dry mass ratio (Turner, 1997; Liu and Stützel, 2004). Drought is known to reduce both root and shoot growth. However Sharp and Davies (1979) found that solutes accumulated at the tip of the root under water deficit, in turn attracting water at these areas which sustain root turgor and growth. Furthermore, under water stress, the deep rooted plant can avoid drought due to its ability to absorb great amounts of water and store it (Levitt, 1980). Exposure of plants to extended drought-stress results in the deeper roots being found in drier soil layers, while the shallower roots are generally found in the wetter soil (Taiz and Zeiger, 1998) The ability of the plant to obtain soil water is therefore determined by the length of the root, its density and diameter. Roots that are thicker last longer and are capable of making more and longer branches and thus elevate root length density and the ability of water uptake (Nguyen *et al.*, 1997). Conservative shoot growth during drought could be advantageous, especially if root growth is promoted, but genotypes that sustain shoot growth during drought may have greater marketability, which is particularly important for leafy vegetable crops (Liu and Stützel, 2004).

As a consequence of drought, the leaves of the plant reach smaller final sizes and also their cytological structure can be changed compared to ones under well-watered conditions (Heckenberger *et al.*, 1998; Grainier and Tardieau, 1998). In response to drought, the leaf area decreases which in turn reduce evaporation while preserving water during drought-stress periods. Specific leaf area is an indicator of leaf thickness and is reduced under water deficit (Marcelis *et al.*, 1998). The different sensitivity of photosynthesis and leaf area expansion in response to soil drying leads to a decrease in specific leaf area in drought-stressed plants. Leaf expansion is affected earlier than photosynthesis by drought-stress (Tardieu *et al.*, 1999). It has been understood that a decrease in specific leaf area may be a way to improve water use efficiency, because thicker leaves have higher density of chlorophyll and proteins per unit leaf area. As a result they have a greater photosynthetic capacity compared to the thinner leaves (Liu

and Stützel, 2004). A decline in transpiration is observed in vegetable amaranth (*Amaranthus spp.*) exposed to soil drying, due to a decrease in stomatal conductance (Masinde *et al.*, 2006).

2.4. History of *Amaranthus*

Amaranthus is a broadleaf ancient crop originating from Latin America where it was domesticated. It was recovered from the wild varieties in Mexico (Early, 1990) and consists of three important group types, the grain, vegetable and ornamental types. The grain type was used by the ancient middle and South American civilisation as an important food crop which consequently added maximum economic value. *Amaranthus* was cultivated by the Aztec, Mayan and the Incas. *Amaranthus*, which the Aztec tribe called huautli, was cultivated 5,000 to 7,000 years ago in Mexico as a food crop and was an important part of their religion and ritual drinks (Early, 1990). Apart from that *Amaranthus* was also used for its rich colour which serves as a dye in their religious rites. *Amaranthus* as a grain crop was grown in a similar way as maize (*Zea mays* L.) by indigenous South American people. Seeds were ground and used to make tamales and tortillas.

The cultivation of this crop was forbidden after the arrival of the Spanish in Mexico in 1500s, since they considered their practice primitive and pagan (Myers, 1996; Tuscon, 2006). Even though the Spanish wanted to destroy the Aztec culture, this crop was rediscovered because of its high nutritional content and its tolerance to unfavourable environmental conditions (Myers, 1996) and is now profitably cultivated in northern India, southern Asia, China, Europe, Africa (Weber, 1987; Ranade *et al.*, 1997) and are widely used as herbs, ornamentals and vegetables (Bostid, 1984). *Amaranthus* cultivation has become popular in many parts of Africa. In South Africa (SA) it is found in all the provinces where it occurs naturally (Jansen van Rensburg *et* al., 2007).

2.5. Taxonomy

Amaranthus is known as amaranth or pigweed and it belongs to a family of Amaranthaceae (Ray and Roy, 2009). It contains approximately 160 genera and 2400 species. Some of these species are trees, while the majority are used as herbs. The Amaranthaceae family is broadly spread in many parts of the world but mostly found in the subtropical and tropical regions. A closely related family to the Amaranthaceae family, which share several characteristics and uses, is the Chenopodiaceae family, which includes beet, spinach and quinoa. The genus *Amaranthus* has approximately 60 species; few are the cultivated type and most are regarded as the weedy type.

2.6. Cultivation

Amaranthus spp. are cultivated for its seeds which are used as grains, and are harvested later after the plant has naturally maturated. The leaves are used as vegetables (Early, 1990) and have the advantage of being harvested throughout the seasons. The seeds of *Amaranthus* are sowed in rows to facilitate cultivation. Seeds must be planted about 4 mm deep whether sowed in the glasshouse or field for good germination. Due to this shallow depth, drying of the soil must be avoided until germination (O'Brien and Price, 1998). They germinate after 4-6 days and thinning may be done when the plant is 2 weeks old (Mingochi and Luchen, 1995). They can be transplanted when plants are 5-10 cm tall within 21 days (Daloz, 1979) and harvesting every 2-3 weeks for a period of one to two months (Fasuyi *et al.*, 2008).

Vegetable amaranth forms flowers and seeds along the stems. The cultivated grain *Amaranthus* has black seed coats unlike the wild and weedy type which has pale-yellow coats (Drzewiecki, 2001). The seeds of amaranth are very small, tan or dark brown in colour, 2 mm long and weigh approximately 1 mg. Maturity of the seeds of grain amaranth can be determined when the seeds are easily separated from the heads upon rubbing between the hands (O'Brien and Price, 1998). *Amaranthus* flowers are not

edible. They produce small (5-10 cm long) flowers but they vary among cultivars and are found to be borne abundantly in axillary spikes (Palada and Crossman, 1999). This crop has the advantage of being unaffected by common soil diseases like nematodes, fungal and bacterial wilt. Pathogens such as damping off, wet rot and insect problems have previously been reviewed (Wilson, 1990).

2.7. Nutritional value of *Amaranthus*

Various *Amaranthus* species are known to possess high nutritional value. Both the seeds and leaves of *A. tricolor* are known to contain protein of unusual high quality and are richer in vitamins and minerals than cereals (Agbetoye and Oyeneye, 2007 and Gorinstein *et al.,* 2007). Protein content in the leaves of *Amaranthus* is 17-19 percent of the dry weight and has an advantage of having a more balanced composition of essential amino acids than other paste such as rice, wheat flour, oats and rye (Ray and Roy, 2009). As a leafy nutritious vegetable it can be further improved to enhance the quality of food for people dependent on it for subsistence (Bostid, 1984). It has many species which are used as leafy vegetables, for example: *A. tricolor, A. tristis* and *A. viridis.* Approximately 100 gram of *Amaranthus* vegetable leaves cooked in the absence of oil makes up 45 percent of the daily vitamin A requirement. When compared to other leafy vegetables like spinach, *Amaranthus* has three times more vitamin C, niacin and calcium. Compared with lettuce, *Amaranthus* contains seven times more iron and twenty times more calcium. The stems, inflorescences and also the leaves can serve as animal feed and as a source of natural red food colorants (Xu and Sun, 2001).

Seeds of *Amaranthus* possess dry matter ranges from 90 to 94 percent, N-substances from 15 to 18 percent, other extracts (fat) from 6 to 8 percent, crude fibre from 3 to 5 percent, ash from 2 to 3 percent and nitrogen free extracts ranging between 60 to 65 percent (Písaříková *et al.*, 2005). A study has been done in the past whereby the oatmeal, whole amaranth flour and amaranth seeds were compared both *in vivo* and *in vitro*, and

it was found that both the *Amaranthus* flour and seeds have increased levels of antioxidants. Flour, starch, bran and oil can be produced from the seed. The flour ground from these seeds blends with wheat or maize flours increases the protein content (Escudero *et al.*, 2005). *Amaranthus* seeds are known to have a high level of lysine, which is two times that of wheat and three times that of maize, and can be consumed with other cereals to give a balanced protein source (Escudero *et al.*, 2005). High levels of lysine, arginine and methionine in *Amaranthus* grains were found by Gorinstein *et al.*, (2002). As a substitution of conventional cereals, a high level of essential amino acids in *Amaranthus* seeds predetermines its usage (Gorinstein *et al.*, 2002). *Amaranthus* flour can also be used in bread, cereals, pasta, pancakes mixes, and snack foods mixed with wheat and corn (Kauffman and Weber, 1990). The seeds of *Amaranthus* contain fibre which is an effective agent against cancer and heart disease and is three times more than wheat and its iron content is five times more than wheat. It also has two times more calcium than ordinary milk. Unsaturated oil and high linoleic acid are also present in the seeds, which is of great importance in human nutrition (Mnkeni *et al.*, 2007).

Amaranthus crop has attracted attention in most of the developing countries, because of its high nutritional value and helps in fighting protein malnutrition. Poor nutrition in children, which can lead to blindness can be reduced with the use of 50 to 100 g of amaranth leaves per day (O'Brien and Price, 1998). It is also given to those recovering from illness or fasting, because the cooked grain is 90 percent digestible (Mnkeni *et al.*, 2007).

Aletor and Adeogun (1995) have previously reported the presence of anti-nutritional components such as nitrates in *Amaranthus*. Some *Amaranthus* can possess toxic levels of nitrates and oxalic acid (Cheeke and Bronson, 1979) and these toxic levels are similar to those found in other vegetables like spinach, beet greens, chard and conventional potherbs (Bostid, 1984). Accumulation of nitrate in plants happens when the plants are grown under drought-stress conditions, especially during a period of heavy nitrate

uptake by the plant. High levels of nitrates lead to formation of carcinogenic nitrosamines and gastric cancer. When the rate of conversion of nitrate to nitrite is higher than that of nitrite to ammonia, toxicity occurs (Sleugh *et al.*, 2001). In the event of absorption into the blood, nitrites interact with haemoglobin which in turn affects the oxygen transport mechanism. This results in a condition known as methaemoglobinanaemia (blue baby syndrome). Therefore, it is of importance to know the levels of nitrate present in the accessions of *Amaranthus* (Mirvish 1983). Oxalic acid in *Amaranthus* accumulates as the plant ages, increased amount of soil fertility and also during water deficit conditions. When consumed by humans it binds minerals such as calcium, making it unavailable for absorption from the digestive tract (Bostid, 1984; Palada and Crossman, 1999). Consumption of large amounts leads to the development of mineral deficiency. Therefore, it is recommended that *Amaranthus* (and many other leafy vegetables) must be boiled before being eaten, because boiling dissolves the oxalic acid (Bostid, 1984).

Red colouration of the stem/leaves is caused by anthocyanin. Anthocyanin is associated with enhanced resistances to abiotic stressors such as drought (Chalker-Scott, 1999), heavy metals (Krupa *et al.*, 1996), UV-B (Reddy *et al.*, 1994; Brandt *et al.*, 1995; Alexieva *et al.*, 2001) opposition to herbivores and pathogens (Coley and Aide, 1989). It acts as a sunscreen against damaging UV-B radiation, and as an antioxidant, which protects the plant under stress. *Amaranthus* leafy vegetables are known to have a greater concentration of antioxidant components than beet, cabbage, leafy lettuce and carrots (Cao *et al.*, 1996; Hunter and Fletcher, 2002; Amin *et al.*, 2006).

2.8. Food habits

2.8.1. Traditional use in Africa

Amaranthus is one of the most important crops in Africa (Palada and Crossman, 1999). The leaves of both the grain and vegetable types can be eaten cooked even though those

grown for vegetable use are known to have better taste than the grain types (Bostid, 1984). Nutritious sprouts can be grown by germinating the seeds, whereas the leaves can be prepared like spinach. In some parts of Africa it is commonly eaten as a leafy vegetable, which can be consumed with other foods or alone. *Amaranthus* can be used in salads (Palada and Crossman, 1999), boiled and mixed with a groundnut sauce or combined with condiments to prepare soup. In the North West, Free State and Limpopo provinces, the tender leaves may be prepared with other ingredients such as onions, tomatoes, peanut butter and spices to enhance their taste. In the Eastern Cape the Xhosa people believe that men who eat *Amaranthus* leaves are unmanly therefore it is mainly eaten by women (Mnkeni *et al.*, 2007). In the Democratic Republic of the Congo they use the leaves to prepare their Caribbean soup called callaloo and in Nigeria it is used as a common vegetable which can be used to prepare all the Nigerian carbohydrate dishes (<http://www.underutilized-species.org>). In Africa *Amaranthus spp* such as *A. tricolor, A. hypochondriacus* (vegetables) and *A. caudatus, A. cruentus* (grain) are the most commonly grown.

Amaranthus spp. is also known as African spinach, Indian spinach, bush greens, green leaf, spinach greens and bonongwe. In SA *Amaranthus* is mostly cultivated as a leafy vegetable. It has several collective names referred to by African people; it is known as *morogo or thepe* (Setswana, Pedi, and Sesotho) and *imifino* (isiXhosa and isiZulu). *Amaranthus* species are also cultivated as ornamentals in other parts of the world (Xu and Sun, 2001). Unfortunately, *Amaranthus* is not widely used in SA due to minimal information and knowledge on the crop's high nutritional values and its easier cultivation practise in comparison to other crops. Therefore it is essential to increase the awareness of *Amaranthus* as a vegetable, as well as the nutritional qualities of the seeds.

2.8.2. Commercial use

Amaranthus has received little research interest to date because of lack of knowledge in the crop's nutritional values, bioavailability of the nutrients, and water and agronomic requirements. *Amaranthus* has potential use in commercial markets where it is packed and sold as whole grain, *Amaranthus* flour (high-bran and low-bran) and also in processed food such as cookies, breakfast bars, snacks and breakfast cereals. The seeds can also be popped and sold as popcorns. Unfortunately the seeds are not consumed in South Africa (Mnkeni *et al.*, 2007). Even though this crop can contribute to food security and can be regarded as a 'cash crop', its demand in the market is minimal but gradually growing. *Amaranthus* grain contains a starch fraction which has potential value in industry and as a food product (Myers, 1996). *Amaranthus* has been exclusively used for seed production in the US and other regions of the world (Jefferson, 1999). With the increase in the human population in Africa, *Amaranthus* leaves can become a valuable source of human nutrition (Chweya, 1985; Gerson, 1991). This green leafy vegetable is popular in some parts of Africa and farmers cultivate it for the canning industry (Haq, 2004). This can improve the economics of farms, feed more families and create jobs which will improve their livelihoods.

2.9. Drought-tolerance of *Amaranthus*

Amaranthus is well adapted to adverse growing conditions such as low nutrient soils, and it also grows in a wide range of temperature and irradiation regimes, and this promotes the possibility of using this leafy vegetable as a nutritious crop in semi-arid regions (Liu and Stützel, 2004). Liu and Stützel (2002) have previously reported on the physiological basis of drought-tolerance in *Amaranthus* genotypes in response to soil drying and indicated that *Amaranthus* reveals a high capacity of osmotic adjustment which guarantees that the plant can continue to function under severe drought-stress conditions. *Amaranthus* can withstand drought better than cotton (*Gossypium* spp.), sorghum [*Sorghum bicolor* (L.) Moench] and corn (*Zea mays* L.) (Johnson and

Henderson, 2002). *Amaranthus* is a C4 plant, and it is capable of using sunlight and nutrients at high temperatures (Zheleznov *et al.,* 1997; Ugas *et al.*, 2008) like sorghum and millets (Weber, 1987).

2.10. Radiation and RAPD technique

Radiation is known to cause changes to the hereditary material of the cell (Jie *et al.*, 1993). Genetic variation in nature is too low to represent mutations which can be integrated into plant breeding schemes. Production of plants with improved traits such as drought-tolerance can be achieved by manipulation of genes that preserve the structure of cellular components or protect and preserve cellular functions under the stress (Valliyodan *et al.*, 2006).

Physical and chemical mutagens can be used in mutation induction and produce genetic variations from which preferred mutants may be selected (Novak and Brunner, 1992). M_1 generation limitation is known to help in comparing the usefulness and efficiency of mutagens. Therefore it is important to select an effective and efficient mutagen in mutation-breeding programs so as to get a high frequency of desirable mutations (Wani, 2009). Mutagens either alter genes or break chromosomes (van der Vyver, personal communication). The level of irradiation dose which causes mutation is induced by ionising radiation (Roy *et al.*, 2006). Ionising radiation was frequently used in mutation breeding to produce variations in plants for crop improvement (Ahloowalia and Maluszynski, 2001). Ionising radiation stimulates heritable chromosomal changes at a specific locus, leading to a variety of lesions in a chromosomal DNA (van der Vyver, personal communication). These changes include deletion, repetition or insertion of a section of the genomic DNA, DNA double-strand breaks, altering, missing or mismatching of nucleotide bases as well as intra- and inter-strand cross-links (Shirley *et al.*, 1992; Tuteja *et al.*, 2001).

Many mutations result in visible phenotypic changes; for instance chlorophyll deficiency, plant stature, pericarp colour, leaf marking, and spike density are most easily identified. On the other hand the occurrence of detecting a phenotypically different plant is not in itself sufficient evidence that there has been a mutation. Therefore, molecular markers can be used to identify genome areas affected by gamma radiation. Another study done by Roy *et al.* (2006) on irradiated *Vigna radiata* (mung bean) showed that the DNA damage after radiation was dose dependent.

Gamma radiation was previously reported at the Atomic Energy Corporation, Pelindaba where *A. tricolor* seeds had been irradiated by radioactivity and selected for improved survival. Seeds of *Amaranthus* were subjected to different irradiation doses (0-100; 200; 300; 400; 500) in order to find an optimal dose that will not distract the subsequent seed germination. It was observed that the best dosage in which the plantlets developed into vigorously growing seedlings was LD-50 (160 Gy). Extensive field and glasshouse screening studies for agronomic and physiological evaluation were conducted over several generations to select drought-tolerance and a number of drought-tolerant mutant lines were selected based on data recorded on the drought-tolerance of plants. After four generations (M2-M5) of agronomic and physiological screening, seven drought-tolerant mutant lines (#2, #5, #6, #19, #550, #554 and #993) were selected for further studies (Slabbert *et al.*, 2004). It is essential for scientist and breeders to have more understanding of the mechanisms and identify heritable traits that the *Amaranthus* plants use to adapt to drought-stress conditions.

Several studies have been performed on intra- and inter-specific genetic diversity and the relationships within the evolution of the *Amaranthus* using isozymes and various DNA markers (Lee *et al.*, 2008). Amplified fragment length polymorphism (AFLP) markers can be used to determine genetic similarities among the *Amaranthus* wild type and their mutant lines, as a result most likely produce viable hybrids.

Random amplified polymorphic DNA (RAPD) markers have been previously used to study the genetic diversity (Chan and Sun, 1997) and phylogenetic relationships among *Amaranthus* species (Ray and Roy, 2009). The RAPD technique can be used for surveying genomic variation because it is quick, requires low labour intensity, inexpensive compared to AFLP (amplified fragment length polymorphism) (Vos *et al.*, 1995) and cDNA RDA (complementary DNA representational difference analysis) (Lisitsyn and Wigler, 1993) techniques. RAPD utilises arbitrary primers and polymorphisms are easily detected. This is followed by scoring the presences or absence of the fragment, then relates to sequence variation due to the insertion, substitution or deletion of the nucleotide (McGregory *et al.*, 2000). However, this technique doesn't distinguish between the homozygote presences of a fragment from its heterozygote compared to (AFLP) marker. AFLP is known to be more reliable than RAPD technique but it is time consuming and laborious. This technique is not only used in genetic diversity, is also widely used in DNA fingerprinting and mapping. The RDA technique modified for cDNA can be followed to characterise the differentially expressed genes between the mutant lines and wild type *Amaranthus*, and identify genes that confer drought-tolerance.

Furthermore, comparing the RAPD technique to other polymerase reaction chain (PCR)-based markers, this technique has the potential of randomly sampling a larger number of loci in a simpler pattern compared to other PCR-based markers (Ray and Roy, 2009; Zhang *et al.*, 2002). This technique also requires no sequence information and no knowledge about certain genes within the targeted taxon (Al-Humaid and Motawei, 2004). RAPD can therefore be used to detect mutations such as point mutations and rearrangements. A meaningful understanding of the amaranth genomes along with their genetic diversity is increasing extensively. Morphological and physiological observations have been well documented but these are poorly inherited identifiers' (Mandal and Das, 2002), because this doesn't provide enough information to distinguish genetic differences among *Amaranthus* genotypes. Rao (2004) has previously employed

RAPD analysis on various plants species relative to development of plant genetic conservation and improvement strategies through biotechnology.

RAPD marker has been used on various plants, e.g *Elymus alaskanus* complex (Poaceae) (Zhang *et al.*, 2002) and *Amaranthus* (Ray and Roy, 2009; Chan and Sun, 1997). Work done by Rogozin and Pavlov (2003) confirms that radiation is directed at any specific locus within the plant genome and the mutation frequencies differ greatly along the nucleotides. These loci might reflect structural and functional features which may be involved in plant growth regulation, plant morphology and senescence processes. However the level of the DNA damage after RAPD analysis is not known unless the RAPD profile is further analysed to the sequence level. Hence, in this study, we look into differences between DNA genomes of the two mutants and the parent using RAPD analysis and to determine if they are associated with the increased drought-tolerance traits observed in the mutant lines. Therefore, molecular analysis coupled with morphological and physiological analysis that will be conducted in this research will be useful to improve drought-tolerance of many other crops as well.

CHAPTER 3

3. Materials and Methods

3.1. Plant material

The plant materials studied were *A. tricolor* wild type and two mutant lines (#2 and #5).

3.1.1. Irradiation of *A. tricolor* plants

Previously, at the Atomic Energy Corporation, Pelindaba in 1999, *A. tricolor* seeds had been irradiated by radioactivity and selected for improved survival under drought-stress conditions. Seeds were subjected to different doses (0-100; 200; 300; 400; 500 Gy) of gamma-radiation in order to induce mutations. The irradiated seed material was germinated in seedling trays in the greenhouse to determine the lethal dose (LD_{50}). For greenhouse screening, seeds were sown in seedling trays in 2:1:1 (peat:sand:vermiculite) soil mixture and Multifeed ™ was applied weekly. The LD_{50} was determined after 6-8 weeks of growth based on germination rate, growth and survival. Optimal dosage in which the plantlets developed into vigorously growing seedlings was LD_{50} of 160 Gy.

3.1.2. Plant selection

Extensive field screening of the mutated plant material for agronomic and physiological evaluation was conducted over several generations to select the mutated lines adapted best for drought conditions. M_1 seedlings were grown under a shade cloth in seedling trays in mist beds and then transplanted to the field after 4 weeks. Self-fertilisation was allowed and these seeds were harvested. Surviving progenies were planted for the next generation (M_2). M_2 plantlets were planted into pots and grown for M_3, M_4 and progressed to M_5 and were tested for early drought-tolerance over a period of 3 years (2000-2003). Selection was based on survivals after re-watering, type of growth after

recovery and growth vigour (plant height, leaf size, internode length and disease occurrences). Seven drought-tolerant lines (#2, #5, #6, #19, #550, #554 and #993) were selected for improved drought-tolerance after 5 generations (M_5) (Slabbert, *et al.* 2004). In the current study only two of these drought-tolerant lines (#2 and #5) were selected for detailed morphological and physiological characterisation. Selection was based on survival after re-watering all the plants.

3.2 Soil selection

A. tricolor plants were grown on 5 different soil types to determine which type of soil promoted the growth of the *Amaranthus* lines. *A. tricolor* wild type, mutant #2 and mutant line #5 seeds were germinated in different types of soils, medium 1 (coconut coir, sand and vermiculite at a 1:1:1 ratio), medium 2 (seedling mix with addition of 4.49g/l Murashige & Skoog, 1962 (MS) as a fertiliser), medium 3 (potting soil enriched with compost containing essential micro and macronutrients, vermiculite and sand at 2:1:1), medium 4 (germination mix, Culterra (Pty) Ltd, SA) and medium 5 (germination mix with addition of MS as a fertiliser). Pots of 9.7 cm in height were used and were placed in the glasshouse. The *A. tricolor* lines were grown at a temperature between 28°C (during the day) and 16 to 19°C (at night).

3.3 Morphological characterisation

3.3.1 Plant growth experiment

A. tricolor seeds were germinated in the germination mix with 4.49g/l MS medium (pH was adjusted to 5.8). The germination tray was divided into 3 rows; one row per line was planted for mutant line #5, mutant line #2 and the wild type. The tray was placed in a temperature-controlled greenhouse with a photoperiod of 12 hr at 28°C. The plants that were used for shoot height measurements were labelled with small, blue rings. The plants were watered every third day with water containing 4.49g/l MS. At 14 days after

emergence, plants were thinned out so that they were 10 to 15 mm apart. During the third week some plants were selected and transplanted individually to 300 g germination mix with 150 ml of MS per pot, while those labelled with a blue rings were left in the germination tray for weekly shoot measurements. After 28 days, seven pots with individual plants per line were exposed to drought-stress while the rest of the plants were continuously watered every second day. Drought-stress was applied by stopping watering for a period of 19 days. During this drought-stress treatment, several parameters were measured. This included shoot length, leaf area, number of leaves per plant, protein content, pot weight, relative water content and fresh/dry weight ratio from both stressed and non-stressed plants. After 19 days of water deficit, the stressed plants were re-watered to observe plant recovery.

3.3.2 Shoot height

Shoot height was measured on a weekly basis using a measuring tape (mm) and increments in plant height were recorded. Measurements were made from the bottom of the stem (where it emerged from the soil) to the tip of the stem. Measurements for the drought-stressed plants were taken every third day during the 19 days of drought treatment.

3.3.3 Leaf area

The first measurements were taken at the third week of growth. Five plants from each of the three different lines were used for determining the leaf area under well-watered and drought-stress conditions. These measurements were taken every third day during drought-stress conditions for a period of 16 days and after 8 days of rehydration. Six to eighteen leaves per plant (depending on the age of the plant) were cut early in the morning and placed on top of a piece of paper and their shapes were drawn and cut out. Each paper shape was weighed and the results were recorded and compared to a piece of paper of known area (standard).

3.3.4 Leaf number

Seven individual plants of each line grown under well-watered and drought-stressed conditions were used for determining the number of leaves per plant. For well-watered plants, the number of leaves per plant was counted weekly during the first 3 weeks of plant growth. For the drought-stressed plants, the number of leaves per plant was counted every third day.

3.3.5 Anthocyanin concentration

Approximately 5-8 stems per line were used for anthocyanin determination after three weeks of plant growth. The leaves were removed from the stems, the weight of the stems recorded and the stems were homogenised in 0.5 ml of 1N hydrochloric acid (HCl) using a mortar and pestle. Homogenised plant extract (0.5 ml) was transferred to 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 13 000 rpm for 5 min after which 0.5 ml of the clear supernatant was transferred to a clean microcentrifuge tube and mixed with 0.5 ml 1N HCl until the solution became turbid. The absorbance of the solution was measured in a spectrophotometer at 515 nm and the absorbance was used to determine the anthocyanin concentration using the following equation:

$$Anthocyanin = \frac{absorbance \times 1\ ml}{grams\ of\ fresh\ weight\ (FW)}$$

3.3.6 Protein determination

3.3.6.1 Protein standard curve

A standard curve was used as a reference in order to provide a relative measurement of protein concentration. The curve was prepared using 10 mg/ml bovine serum albumin (BSA) from Sigma-Aldrich (United States of America) as a stock solution and distilled

water. A dilution series of 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml BSA solution was prepared and the BioRad protein assay was used for protein determination (BioRad Laboratories, California). Ten µl from each dilution was added to 790 µl distilled water and 200 µl of the BioRad protein assay reagent. The solution was mixed and the absorbance was measured in a 1.5 ml cuvette at 595 nm using a spectrophotometer (Macince, UK).

3.3.6.2 Protein concentration of leaves

The third leaf from 21-day old, well-watered plants of each line was cut from the plants and its weight recorded. During drought-stress one leaf from each plant was removed and cut in half. One half was used to determine its fresh weight/dry weight (FW/DW) ratio while the other half was used for determining the protein concentration. Protein measurements were performed every third day from well-watered and drought-stressed plants during drought conditions. To ensure that the size of the leaves was similar, approximately an equal size of leaf was cut at each time point. During 19 days of drought treatment, the protein was determined at three day intervals. Leaves of the same age from seven plants per line were used.

The leaves were homogenised individually in 1 ml 50mM Tris-HCl buffer, pH 8.0. Ten µl of each sample was added to 790 µl dH_2O and 200 µl BioRad protein assay reagent. The samples were mixed well and left at room temperature for 5-10 min and the absorbance was measured in a spectrophotometer at 595 nm.

Protein concentration was determined using the formula: $y = mx + c$

'x' and 'y': coordinates of a straight line graph

'm': the gradient of the straight line graph

'c': is the intercept of the straight line graph

$$\text{Protein content (g)} = \frac{\text{gram protein X } 0.5 \text{ ml}}{\text{grams of fresh weight (FW)}}$$

3.3.7 Fresh weight and dry weight

During 19 days of well-watered and drought-stress conditions, FW and DW were determined from leaves of the same age from seven plants per line. The fresh leaves were weighed to determine the FW values. This was followed by drying the leaves overnight at 70°C. The dried leaves were weighed again in order to obtain the DW values.

3.3.8 Relative water content (RWC)

The RWC was determined every third day of the drought-stress period using six plants per line. Immediately after harvesting, leaf disks (10 mm in diameter) were weighed to obtain FW. The samples were rehydrated in 3 ml of distilled water for 4 hrs. Thereafter the leaf disks were patted dry on paper towel and weighed again to obtain the turgid weight (TW). The leaf samples were oven-dried overnight at 70°C, and weighed again to obtain DW. FW, TW and DW data for each leaf disc were recorded and used to determine the RWC and the FW/DW ratio. RWC was calculated following a methodology described by Turner (1981) using the formula:

$$RWC = [(FW-DW) / (TW- DW)] X 100$$

3.3.9 Pot weight during drought-stress

The weight of each pot of all 21 plants (seven pots per line) under drought-stress was determined using a weighing balance (Microsep (Pty) Ltd, SA). These measurements were made every third day during the 19 days of drought-stress.

3.4 RAPD analysis

3.4.1 Isolation of DNA

Four individual *A. tricolor* leaves per line were harvested and their weights recorded before isolating their DNA. DNA was isolated from these leaves using a Qiagen DNA Purification Kit for total DNA from plant tissue (Qiagen DNeasy Plant Handbook, 2006). The concentration of the DNA samples was measured at 260/280 ratio using the ND-1000 Nanodrop® spectrophotometer (Nanodrop Technologies, USA).

3.4.2 Polymerase Chain Reaction (PCR)

Random Amplified Polymorphic DNA (RAPD) analysis was performed in order to detect the differences in DNA of non-mutated wild type and the mutant lines. Polymerase chain reaction (PCR) for amplification of DNA was performed using a MyCycler™ thermal cycler (BioRad) and RAPD primers. Nineteen arbitrary primers (namely OPA-01, OPA-17, OPB-01 and OPB-02 (Operon Technologies, USA)) (Table 1) were tested for detection of polymorphisms. The PCR reactions were performed in 0.2 ml microcentrifuge tubes which contained 3.5 mM $MgCl_2$, 0.04 mM dNTPs, 0.24 µM primer, 0.75 U TaKaRa *Taq* polymerase and 30 ng genomic DNA in a final volume of 16 µl. DNA was amplified using MyCycler™ thermal cycler (BioRad) under the following parameters: denaturing step of 1 min 30 sec at 94.5°C followed by 35 cycles consisting of 15 sec at 94.5°C, 20 sec at 37°C, 30 sec at 72°C with a final DNA extension of 2 min at 72°C followed by 2 min at 10°C to terminate the reaction.

3.4.3 Gel Electrophoresis

The DNA samples were separated using a 1.2 percent agarose gel containing 1X TAE (Tris-Acetate-EDTA) buffer. The gel was run for 90min at 80 V and stained in 250 ml of deionised water and 10µl of ethidium bromide for 10-15 min. The result of the gel was visualised using UV light on a transilluminator. The reactions were repeated three times

without alteration in the protocol in order to verify the reproducibility of the amplification pattern.

Table 1: Nucleotide sequences of primers used in RAPD analysis

Primer	Sequence
OPA-01	5'-CAGGCCCTTC-3'
OPA-02	5'-TGCCGAGCTG-3'
OPA-03	5'-AGTCAGCCAC-3'
OPA-04	5'-AATCGGGCTG-3'
OPA-05	5'-AGGGGTCTTG-3'
OPA-06	5'-GGTCCCTGAC-3'
OPA-07	5'-GAAACGGGTG-3'
OPA-08	5'-GTGACGTAGG-3'
OPA-09	5'-GGGTAACGCC-3'
OPA-10	5'-GTGATCGCAG-3'
OPA-11	5'-CAATCGCCGT-3'
OPA-12	5'-TCGGCGATAG-3'
OPA-13	5'-CAGCACCCAC-3'
OPA-14	5'-TCTGTGCTGG-3'
OPA-15	5'-TTCCGAACCC-3'
OPA-16	5'-AGCCAGCGAA-3'
OPA-17	5'-GACCGCTTGT-3'
OPA-18	5'-AGGTGACCGT-3'
OPA-19	5'-CAAACGTCGG-3'
OPB-01	5'-GTTTCGCTCC-3'
OPB-02	5'-TGATCCCTGG-3'

3.5 Statistical analysis

The results of shoot length, leaf area, anthocyanin content, protein content, RWC and FW/DW ratio were analysed using the General Linear Model (GLM) of the Statistical Analysis System (SAS Institute Inc. Cary, NC, United States 1996). Statistical analysis was performed by using the student's t-test with a significance level of 5 percent.

CHAPTER 4

4. Results and Findings

4.1. Morphological and physiological characterisation of mutated *A. tricolor* lines during well-watered conditions

4.1.1. Selection of optimal soil medium

Preliminary experiments were performed under well-watered conditions using five different types of soil in order to determine which soil type results in optimal growth for all the plants. Medium 1 (coconut coir, sand and vermiculite at a 1:1:1 ratio) resulted in the loss of leaves after 25 days and eventually some plants died. Medium 2 (seedling mix with addition of 4.49g/l Murashige & Skoog (MS) as a fertiliser) favoured the growth of the plants, but the soil contained stalks, which seemed to interfere with seed germination and subsequent plant growth. A white surface layer (salt precipitation) developed on medium 3 (potting soil enriched with compost containing essential micro and macronutrients, vermiculite and sand at 2:1:1) after 3 weeks of plant growth and growth of the plants was very slow. Medium 4 (germination mix, Culterra (Pty) Ltd, SA) did not provide enough material for plant extraction after 4 weeks. The plants were very small possibly due to certain nutrients not being provided to enhance plant growth. Therefore, MS salt mixture was added into the soil mixture as a fertiliser (medium 5). Medium 5 (germination mix and MS) allowed good growth of plants, since the third leaf of the plants appeared already during the first week of growth. Furthermore, the texture of the soil did not have any negative effect on the germination of the seeds. The wild type and mutant #5 plants performed the best regarding plant growth when medium 5 was used (Figure 4.1). As a result, medium 5 was subsequently used for the cultivation of plants.

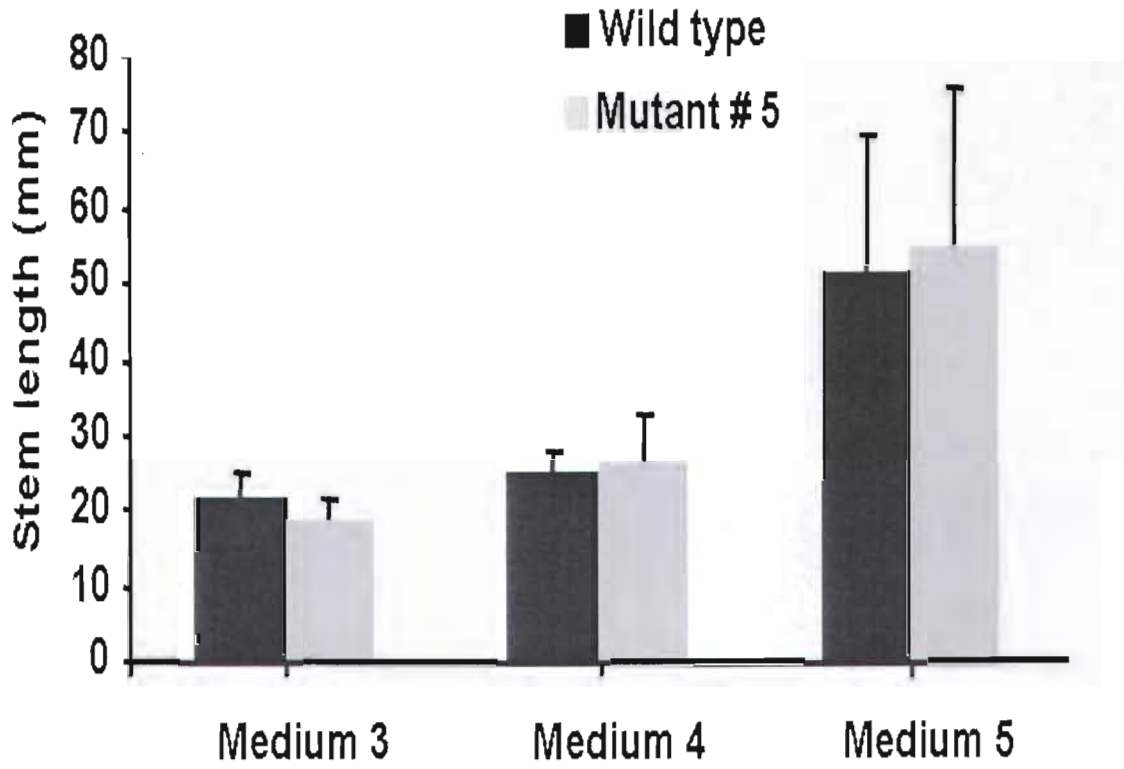

Figure 4.1: Growth comparison of 10 *Amaranthus* plants on different soil types (medium3 (potting soil, vermiculite and sand), medium 4 (germination mix) and medium 5 (germination mix with MS)) after 4 weeks of growth. Data shown are the mean from 10 plants of each line ± SE (standard error).

4.1.2. Plants grown

Amaranthus tricolor plants used for shoot length measurements were labeled with blue rings (Figure 4.2).

Shoot lengths for all the *Amaranthus* lines were measured weekly over a period of 5 weeks. This experiment was repeated four times due to differences in natural light conditions. Mutant #5 grew slightly faster when compared to mutant #2 and the wild type, but the difference was not significant statistically (P>0.05) (Figure 4.3).

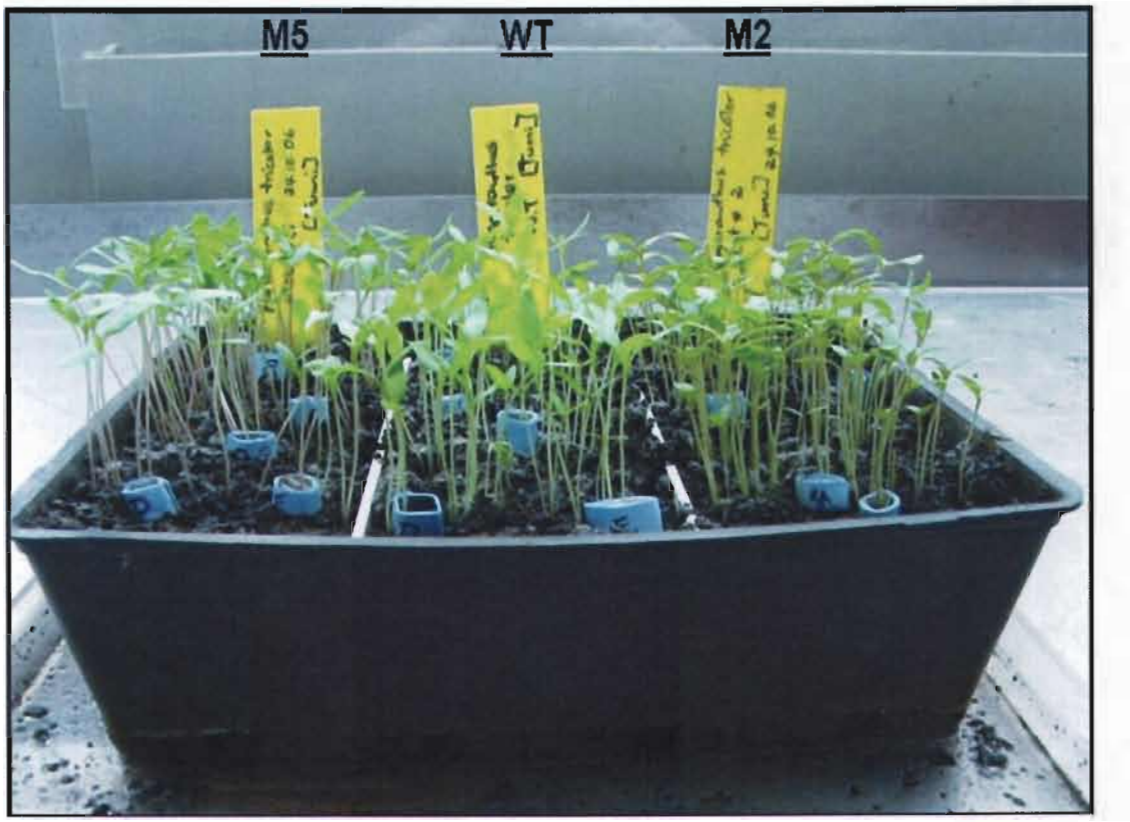

Figure 4.2: Two week old plants of *Amaranthus* lines (wild type, mutant #2 and mutant #5). Plants labeled with blue rings were the plants used for weekly measurements.

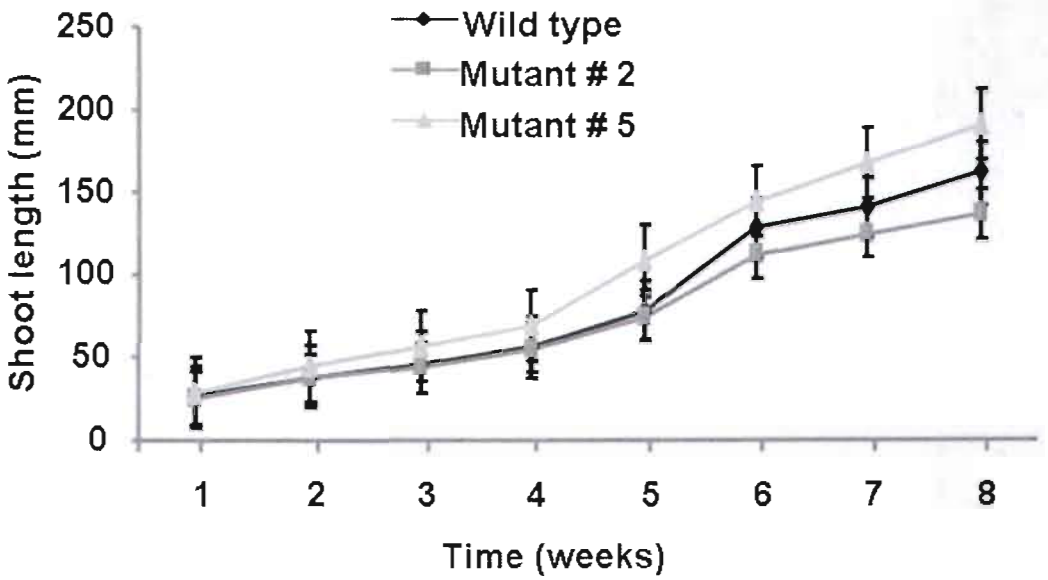

Figure 4.3: Average shoot length of 7 plants from the wild type, mutant #2 and mutant #5 under well-watered conditions in the greenhouse at 28 °C. Data shown are the mean from 7 plants of each line ± SE at each time point.

4.1.3 Anthocyanin content

The anthocyanin content from the stems of the *Amaranthus* wild type as well as the mutant lines was determined after three weeks of plant growth. The difference in stem colour became visible during the second week of plant growth. The stems of mutant #2 (light pink), mutant #5 (light and dark pink) and wild type (green) were significantly different ($P<0.05$) in colour after three weeks of growth (Figure 4.4 A). But there was no significant difference ($P>0.05$) found between the two mutant lines compared to the wild type plants (Figure 4.4 B).

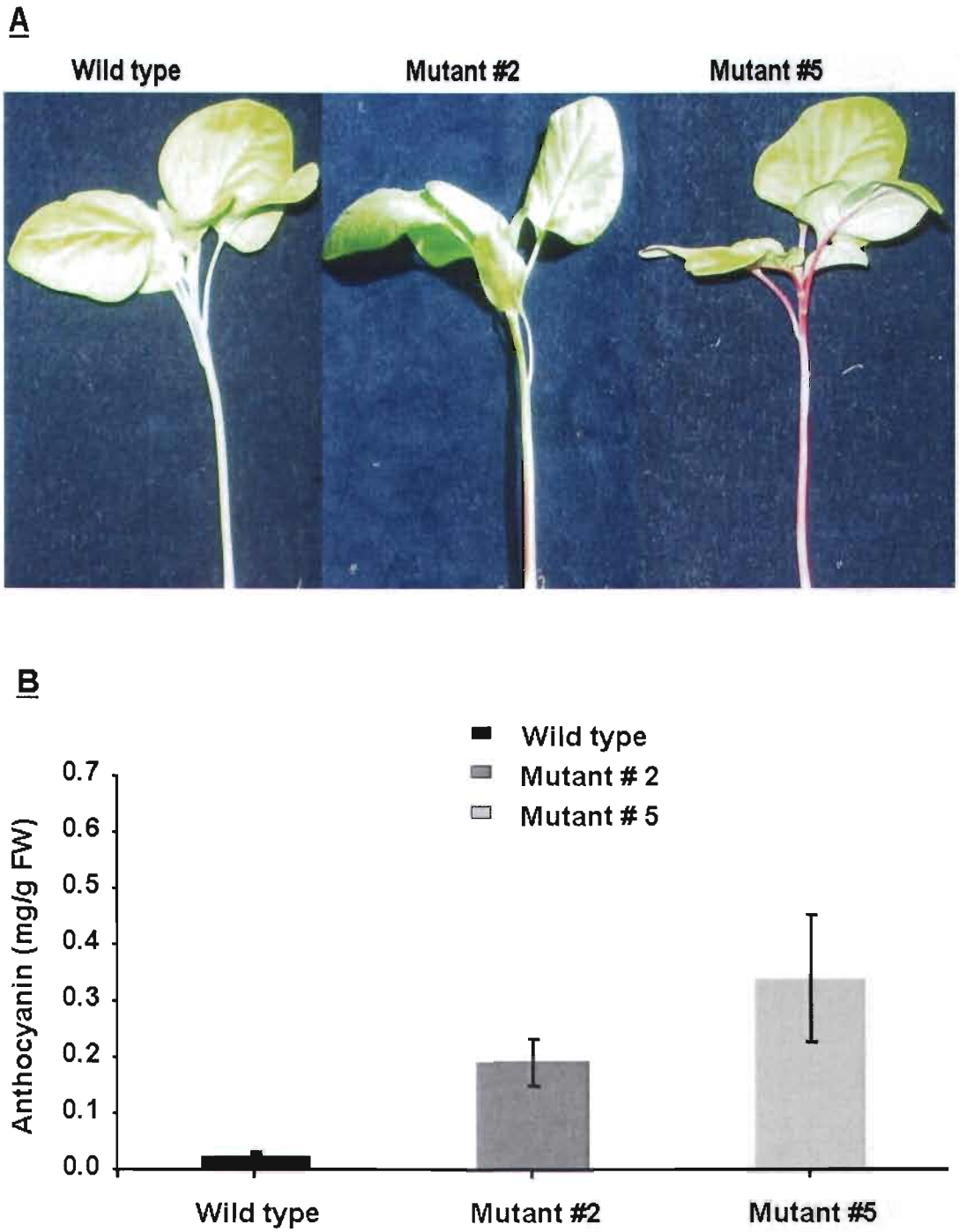

Figure 4.4: (A) Stem colour of the wild type, mutant #2 and mutant #5 plants and (B) the average concentration of the anthocyanin content from these lines after 3 weeks of growth at 28°C. Data shown are the mean from 8 stem stalks of each line ± SE.

4.2. Morphological and physiological characterisation of *Amaranthus* lines during well-watered and drought-stress conditions

4.2.1. Effect of drought-stress on shoot length

Under well-watered conditions, the shoot lengths of mutant #5 plants were significantly higher ($P<0.05$) than of mutant #2 and the wild type plants (Figure 4.5 A). The shoot lengths of the wild type plants were not significantly different from mutant #2 plants ($P>0.05$). When plants were exposed to 19 days of drought conditions, there was a significant reduction ($P<0.05$) in shoot length in comparison to well-watered plants. The shoot length of mutant #5 decreased by 27 percent, the wild type by 21 percent and mutant #2 by 18 percent when compared to well-watered plants. However, mutant #5 plants still grew faster than mutant #2 and the wild type plants under drought-stress conditions and a significant difference in growth was observed after 19 days ($P<0.05$) (Figure 4.5 B).

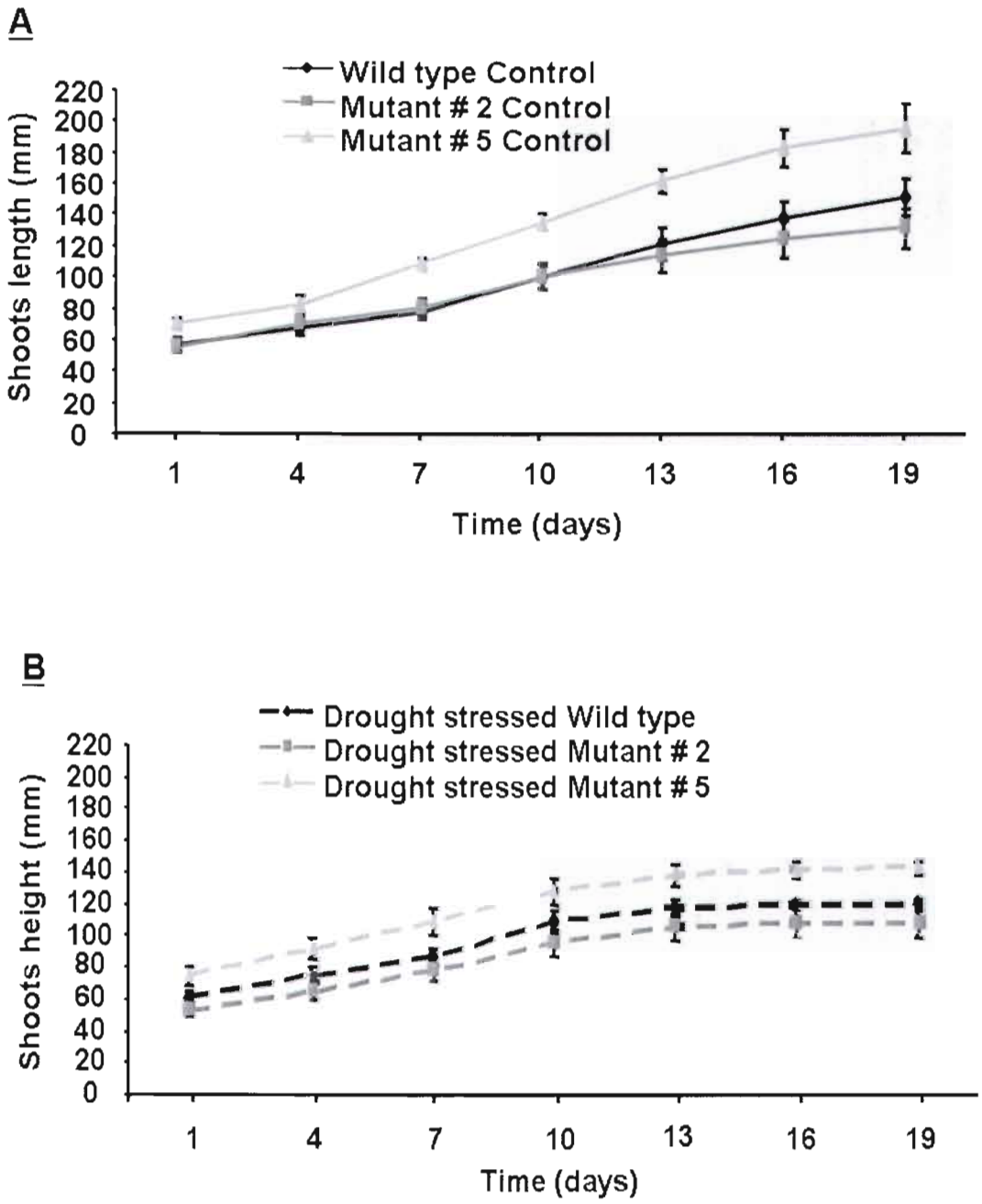

Figure 4.5: (A) The average shoot length of seven wild type, mutant #2 and mutant #5 plants during 19 days of (A) well-watered and (B) of drought-stress conditions in the greenhouse at 28°C. Data shown are the mean from seven plants of each line ± SE at each time point.

4.2.2. Leaf area

The leaf area per plant was measured from both well-watered and drought-stressed plants. During well-watered conditions, the leaves of the wild type plants were bigger when compared to both mutant lines and this was observed consistently over a period of 24 days (Figure 4.6 A). After 24 days, however, the leaf areas of the wild type plants were not significantly different ($P>0.05$) from leaf areas of the mutant lines.

After 16 days of drought-stress, the total leaf area of plants was significantly reduced when compared to well-watered conditions. The leaf areas of the wild type plants were reduced by 77 percent and the leaf areas of both mutant lines by 87 percent ($P<0.05$; Figure 4.6 B). Thus, at 16 days of drought-stress the wild type plants had larger leaf areas than the leaf areas of both mutant lines, but this difference was not significant ($P>0.05$). After 16 days of drought treatment, all leaves of wild type plants dried out and did not recover after re-watering. Plants of both mutant lines, however, recovered after re-watering and the leaf areas significantly increased during an 8 day re-watering period (Figure 4.6 B).

4.2.3. Number of leaves

Wild type plants had more leaves under both well-watered and drought-stress conditions when compared to plants of both mutant lines, which had similar numbers of leaves under both conditions (Figure 4.7 A). Although plants of all three lines developed less leaves during drought-stress and exhibited a delay in leaf development, this decrease in leaf number was not significant when compared to well-watered plants (Figure 4.7 B). The number of leaves of wild type decreased by 17 percent, the mutant #5 by 10 percent and mutant #2 by 8 percent when compared to well-watered plants.

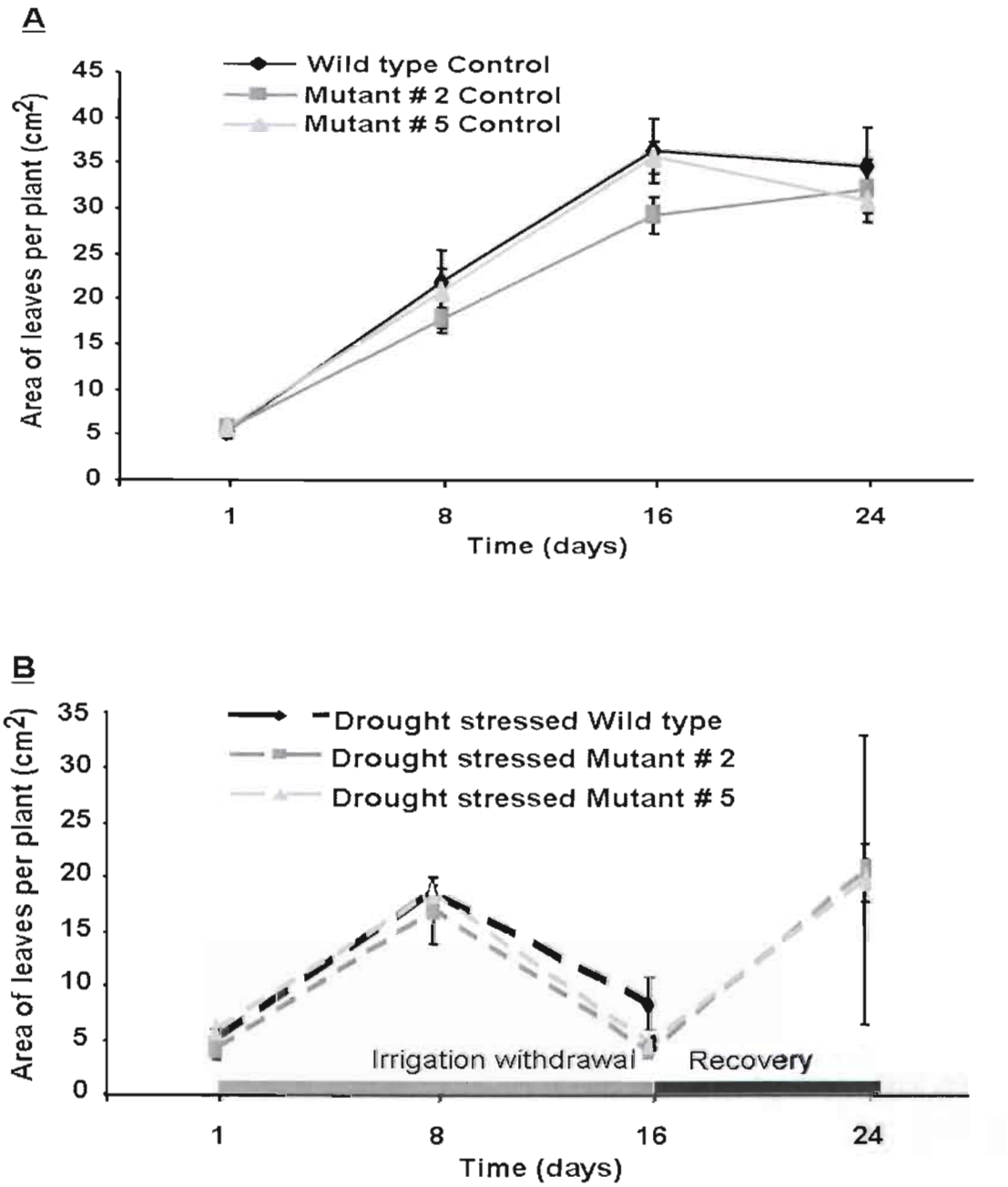

Figure 4.6: (A) The average leaf area of wild type, mutant #2 and mutant #5 plants during 24 days of well-watered conditions and (B) during 16 days of drought conditions and eight days of recovery period in the greenhouse at 28°C. Data shown are the mean from five plants of each line ± SE at each time point.

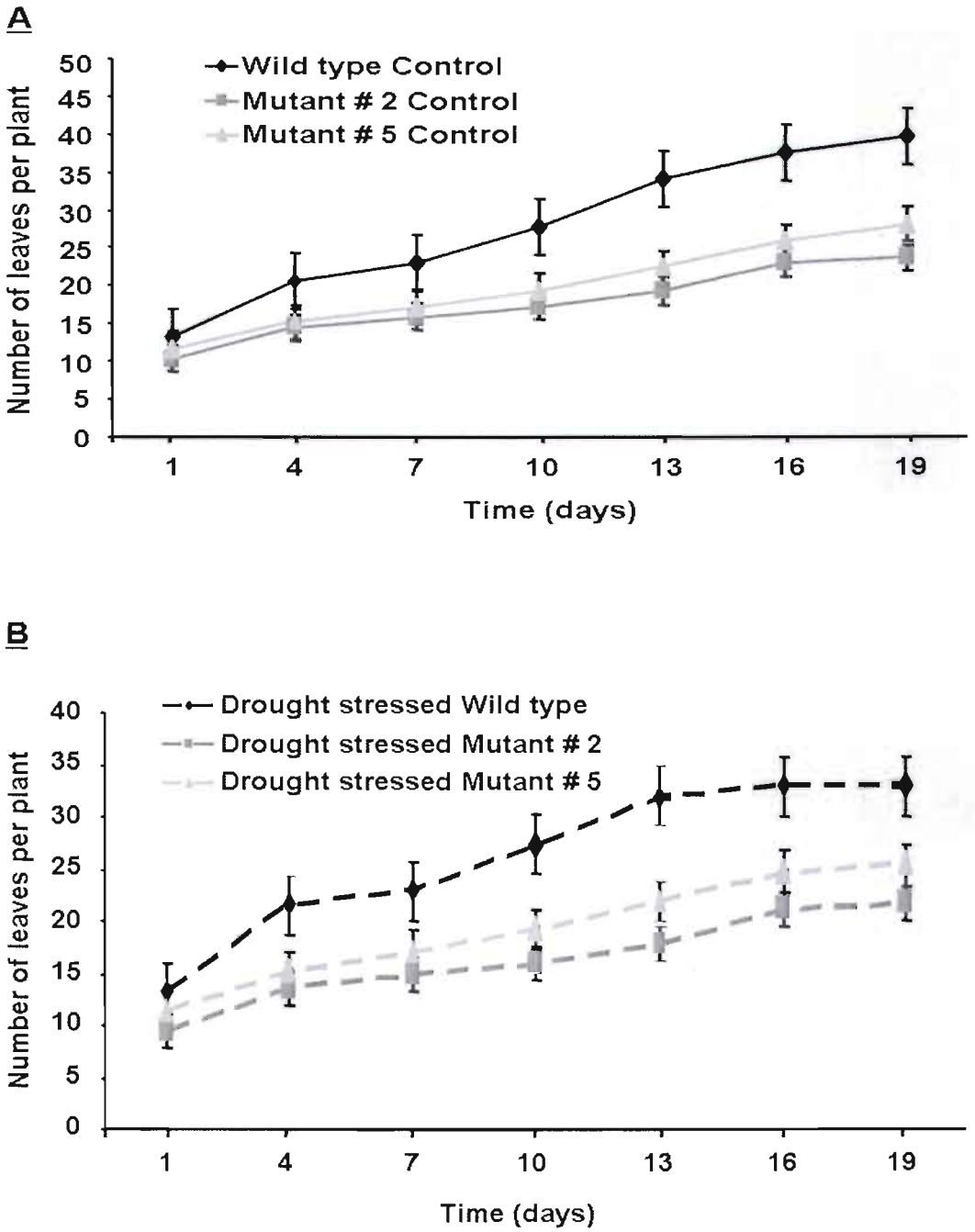

Figure 4.7: (A) The average number of leaves from wild type, mutant #2 and mutant #5 per line during 19 days of (A) well-watering and (B) of drought treatment in the greenhouse at 28°C. Data shown are the mean from seven plants of each line ± SE at each time point.

4.2.4. Protein content

Under well-watered conditions, all plants of all the lines exhibited an increase in protein content per dry weight over time (Figure 4.8 A). When exposed to drought-stress conditions, the protein content started to decrease after 13 days (Figure 4.8 B). Mutant #2 plants had a significantly higher ($P>0.05$) protein content after 19 days under both well-watered and drought-stress conditions when compared to mutant #5 and wild type plants (Figure 4.8 A and B). The protein content was not significantly different between the two mutant lines ($P>0.05$). Drought-stress significantly reduced ($P<0.05$) the protein content in all lines: the protein content of mutant #2 was reduced by 45 percent, mutant #5 by 48 percent and the wild type by 63 percent.

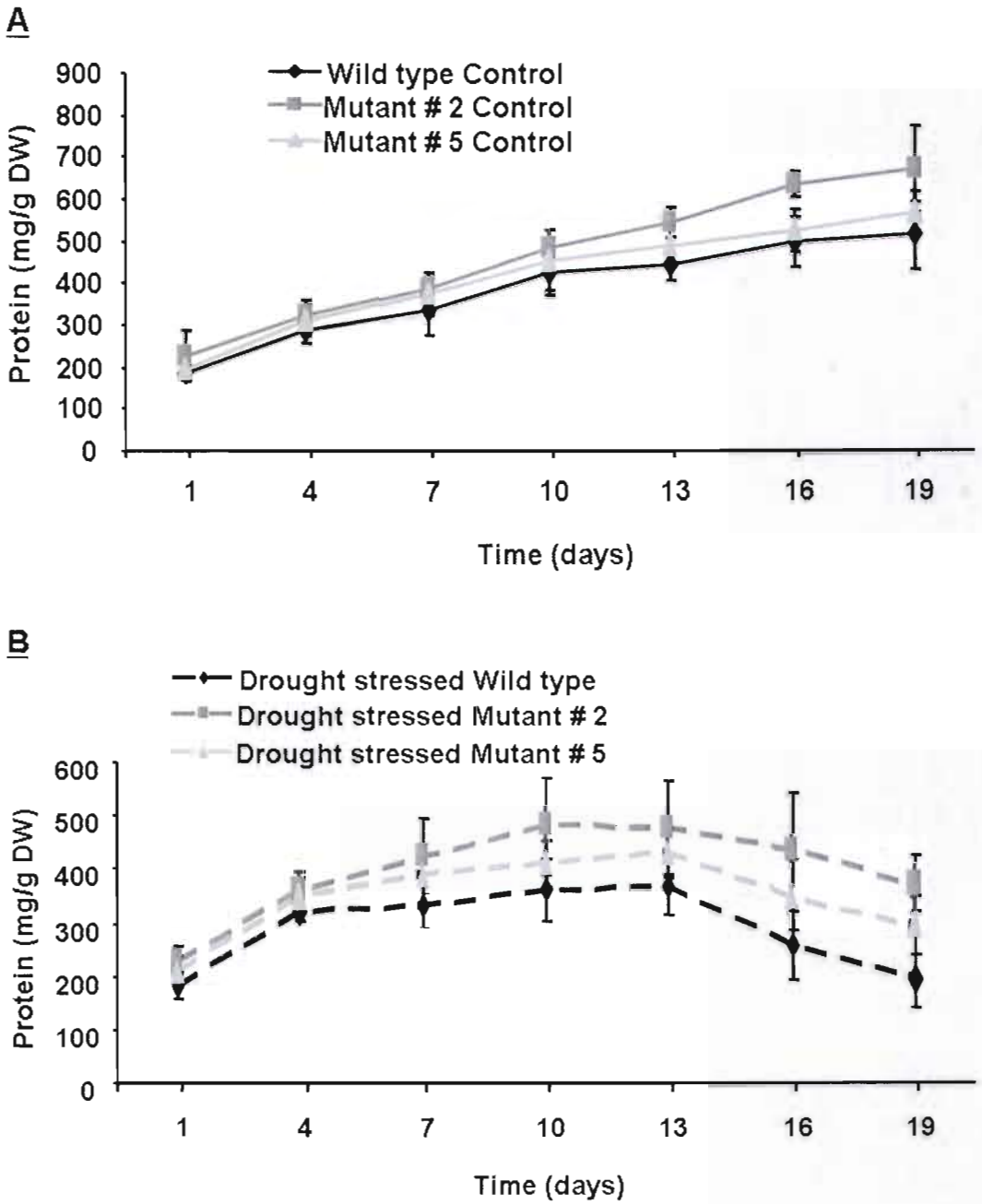

Figure 4.8: A) The average protein content from the wild type, mutant #2 and mutant #5 plants during 19 days of (A) well-watering and (B) of drought treatment in the greenhouse at 28°C. Data shown are the mean from seven plants of each line ± SE at each time point.

4.2.5. Relative water content, FW/DW ratio and pot weight

To monitor the progression of drought-stress the weight of the pots where the plants were planted (soil + plant biomass weight) was measured over 19 days (Figure 4.9). The pot weight progressively decreased as the treatment period progressed, but there was no significant difference in the decrease between the pots containing the three different lines ($P<0.05$).

Both the RWC and the FW/DW ratio of all three lines were affected by drought-stress (Figures 4.10 and 4.11 B). After 19 days, wild type plants lost slightly more water than plants of both mutant lines, but the difference in RWC was not significant ($P>0.05$; Figure 4.10). After the drought-stress period, the RWC of the wild type plants was reduced by 69 percent, the mutant #2 plants by 72 percent and the mutant #5 plants by 75 percent when compared to the RWC at the beginning of the drought period. In addition, the FW/DW ratio greatly decreased as the drought period progressed and all lines exhibited a significantly lower FW/DW ratio ($P<0.05$; Figure 4.11 B). However, there was no significant ($P>0.05$) difference in the decrease of both RWC and FW/DW ratio. Meanwhile under well-watered, the FW/DW ratio was not significantly ($P>0.05$) different between all three lines (Figure 4.11 A).

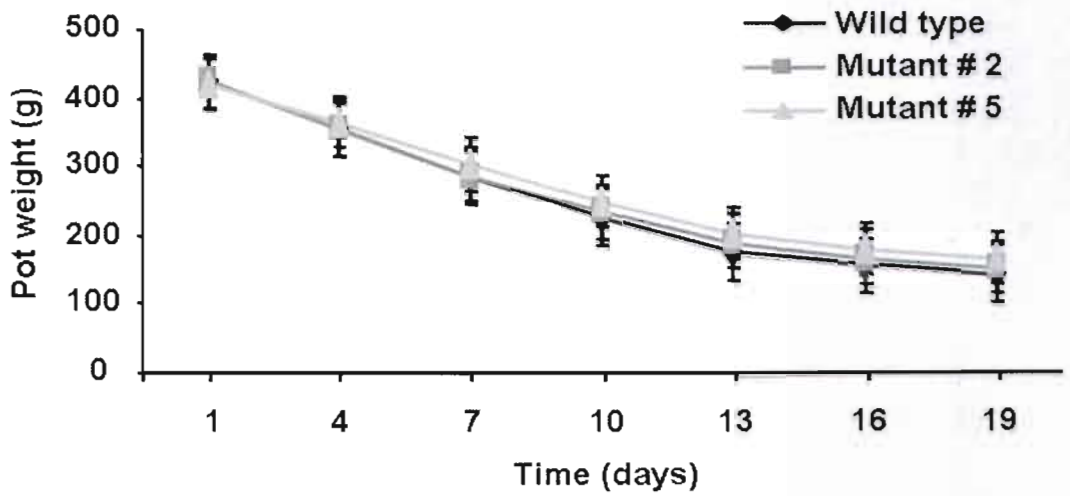

Figure 4.9.: The average weight of the pots (soil and plant biomass weight) containing the wild type, mutant #2 and mutant #5 plants during 19 days of drought treatment in the greenhouse at 28°C. Data shown are the mean from seven pots for each line ± SE at each time point.

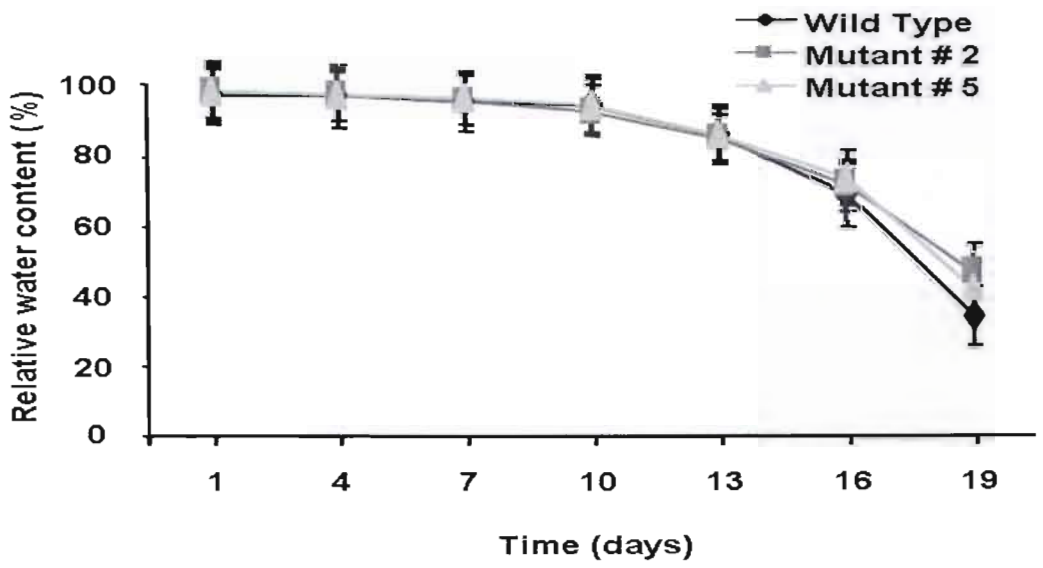

Figure 4.10: Relative water content (RWC) of the wild type, mutant #2 and mutant #5 plants during 19 days of drought treatment grown at 28°C in the greenhouse. Data shown are the mean from six plants of each line ± SE at each time point.

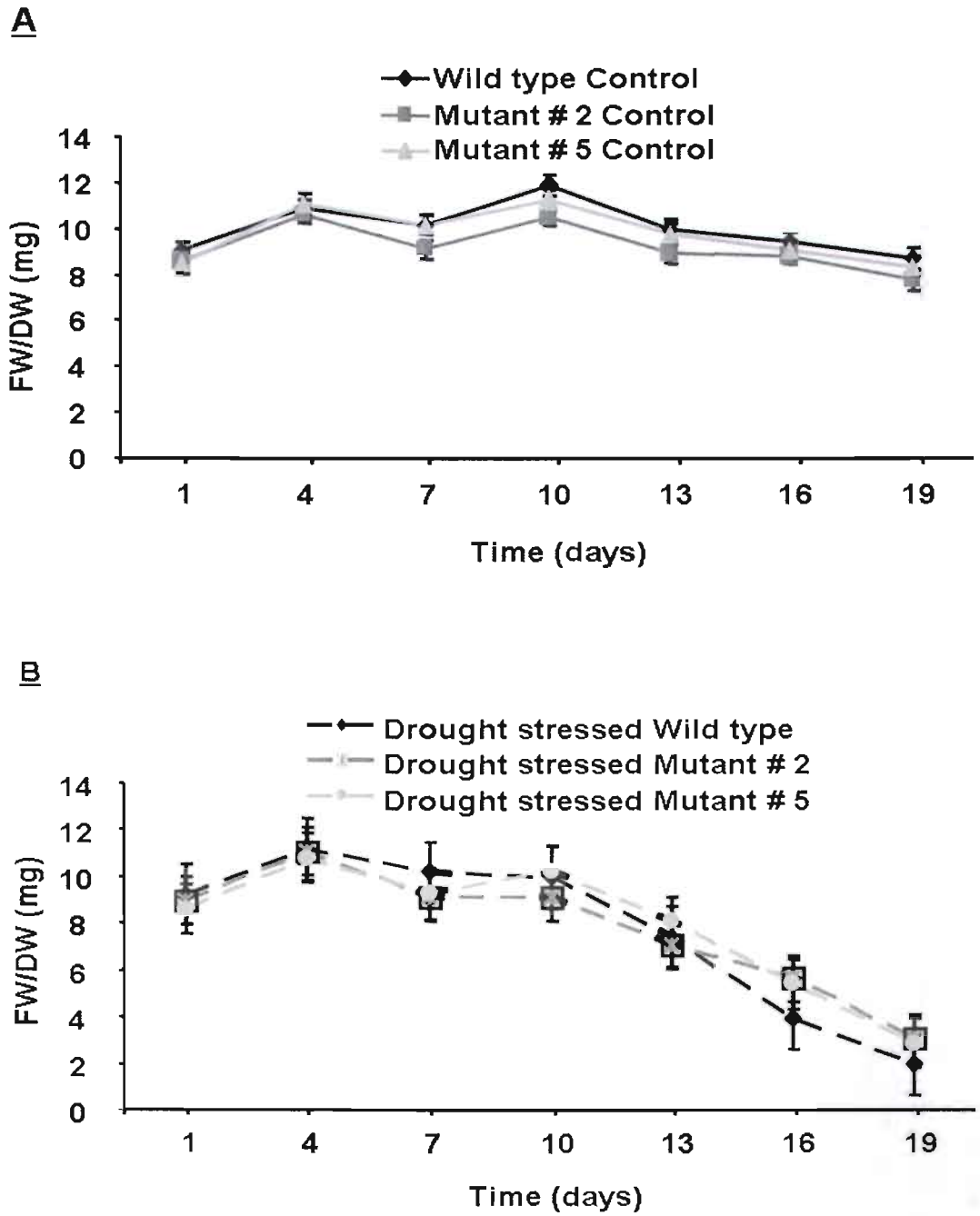

Figure 4.11: The average fresh weight/dry weight (FW/DW) changes of the wild type, mutant #2 and mutant #5 plants during 19 days of (A) well-watering and (B) of drought treatment in the greenhouse at 28°C. Data shown are the mean from seven plants of each line ± S.E at each time point.

4.2.6. Recovery of plants after re-watering

After 13 days of drought-stress the plants started to show symptoms of water deficit. This included loss of chlorophyll in the lower leaves, while others orientated their leaves away from the sun. Signs of wilting were also obvious in all eight wild type plants, while wilting was only observed in four mutant #5 and two mutant #2 plants (data not shown). After 19 days of drought-stress, all wild type plants were dead, whereas leaves of mutant #2 and mutant #5 plants exhibited yellowing (Figure 4.12 A).

At the end of the drought-stress period, none of the wild type plants recovered when re-watered (Figure 4.12 B). Out of all seven plants used per line, mutant #2 plants had a maximum of four plants surviving and mutant #5 plants had a maximum of three plants surviving and they started growing after re-watering (Figure 4.12 B). Furthermore, after seven days of re-watering, plants of both mutant lines started flowering.

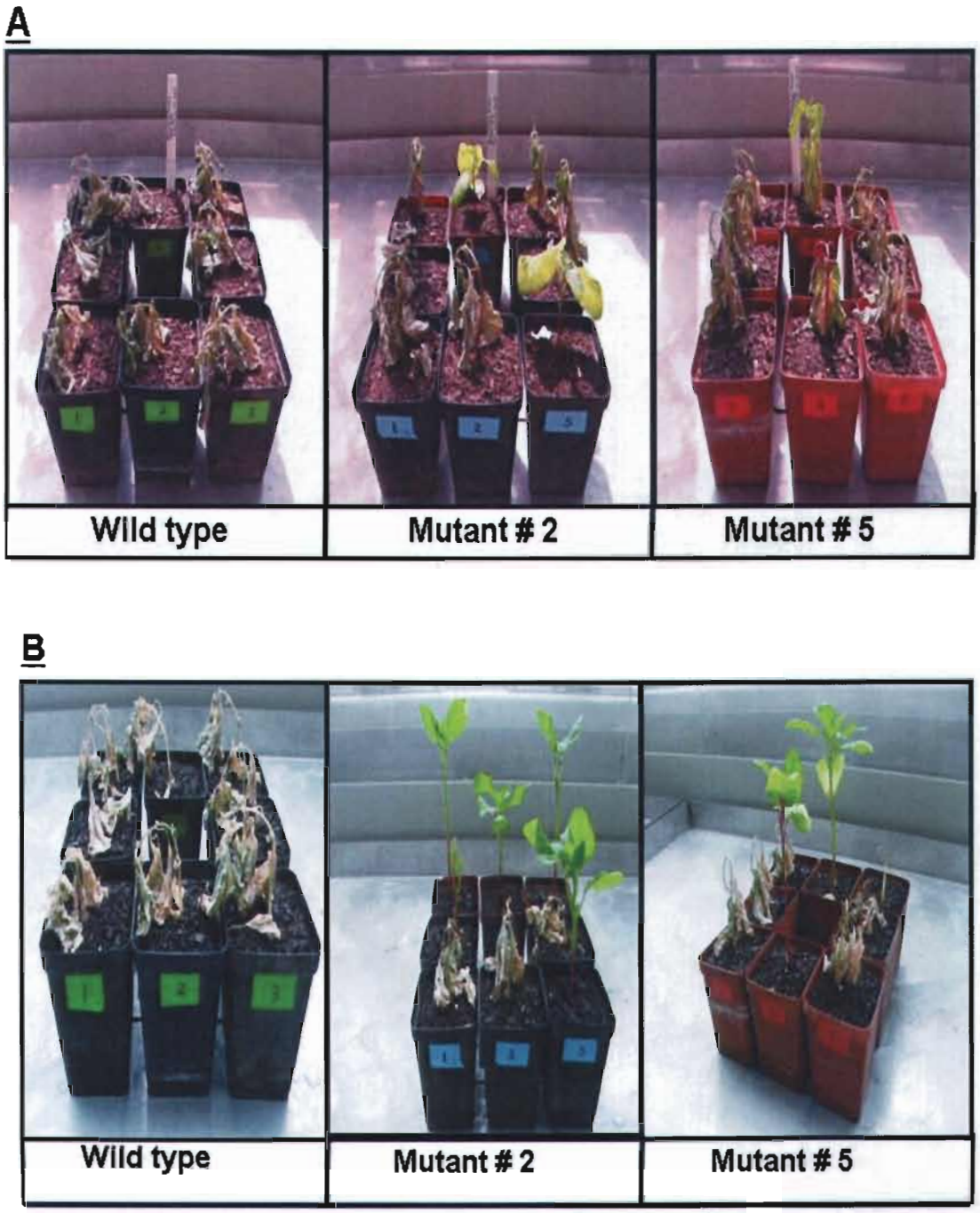

Figure 4.12: (A) Physical appearance of the wild type, mutant #2 and mutant #5 after 19 days of growth under drought-stress conditions and (B) after seven days of re-hydration in the greenhouse at 28°C.

4.3. Detection of DNA polymorphisms using RAPD analysis

In order to detect polymorphisms between the genomic DNA of the wild type and mutant lines, random amplified polymorphic DNA (RAPD) analysis was performed. Nineteen arbitrary primers were used, but only two (OPA-07 and OPA-16) detected differences between the three lines. An analysis of four plants from each line was conducted with selected primers and as a result no variations were observed within these four plants from the same line using OPA-07 and OPA-16 primers. Primer OPA-07 detected a polymorphism (A1; Figure 4.13 A) between mutant #2 and mutant #5 (the fragment observed in mutant #2 was also present in the wild type). Primer OPA-16 detected a polymorphism between the wild type and mutant #2 (A2; Figure 4.13 B). The fragment detected in mutant #2 was also not present in mutant #5. This fragment would be the most useful to use during further studies, since it is present in one of the drought-tolerant lines, but not in the wild type.

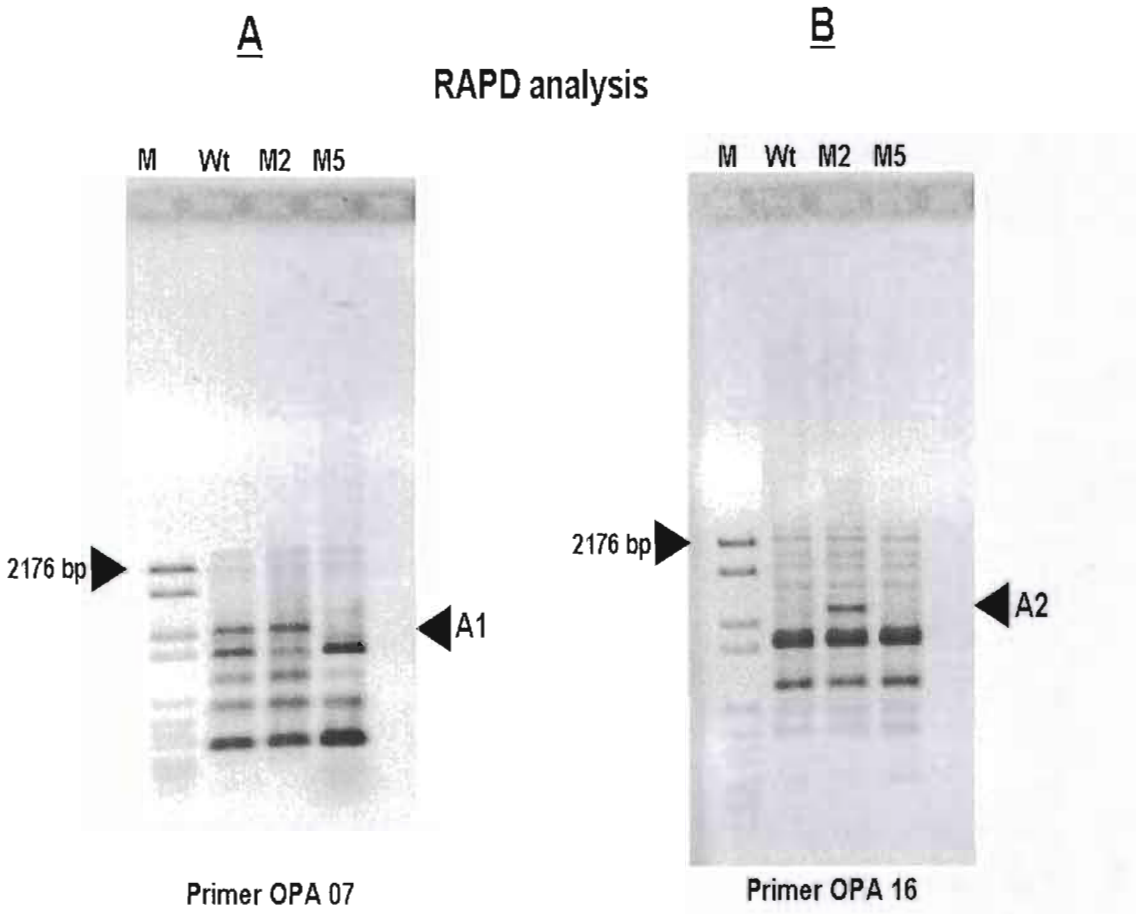

Figure 4.13: RAPD analysis of genomic DNA of the wild type, mutant #2 and mutant #5 plants after visualisation of the amplified DNA fragments on a 1.2 percent agarose gel. M represents Molecular Weight Marker IV; Wt represents the wild type; M2 represents mutant #2 and M5 represents mutant #5. (A) represents amplification using OPA-07 and (B) represents amplification using OPA-16.

CHAPTER 5

5. Discussion

5.1. Morphological and physiological studies

The current study focussed on two irradiated and one non-irradiated *A. tricolor* lines. These lines were characterised to determine the effect of drought on the mutated material, as well as to determine which one of the two mutant cultivars is the most drought-tolerant. The mutant lines were produced by irradiating the *A. tricolor* seeds using gamma radiation. M_1 seedlings were planted in the field and in wooden boxes in the greenhouse to screen for drought-tolerance (Slabbert *et al.*, 2004). The M_2-M_5 generations from these selected lines were tested for early drought-tolerance over a period of three years from 2000-2003. After four generations of agronomic and physiological screening, seven drought-tolerant mutant lines (#2, #5, #6, #19, #550, #554 and #993) were selected. For this study, lines #2 and #5 were selected for more detailed analysis.

The current study indicated that physiological and biochemical characteristics of the *Amaranthus* lines were altered by drought-stress, in particular there was a decrease in plant length, leaf area, number of leaves, protein content and FW compared to well-watered plants. The results also indicated that both mutant lines retained more water under drought conditions compared to the wild type, with mutant #2 performing better than mutant #5.

Generally, plant development is enhanced by the availability of soil water for shoot development and growth. Various internal (gibberellins plant hormones) and external factors (light and temperature) influence plant growth besides its genetic makeup (Sestak *et al.*, 1971). Since shoot growth depends on cell division, enlargement and differentiation, exposure to drought-stress can affect plant growth compared to well-

watered plants (Patel and Golakia, 1988). In our study, the shoot height in all the *Amaranthus* lines was significantly reduced by drought when compared to the well-watered lines. This decrease in shoot growth might be associated with a decline in cell growth and enlargement (Manivannan *et al.*, 2007).

A decrease in soil water potential causes a decrease in leaf area, wilting, impairment of metabolic functions and limitations of photosynthetic activities (Kramar and Boyer, 1983). Leaf area plasticity is of importance in order to control water utilisation in crops (Manivannan *et al.*, 2007). In this study the leaf area of all *Amaranthus* lines were reduced significantly by drought-stress compared to well-watered plants. However, wild type drought-stressed plants still produced bigger leaves than the mutant lines; as a result, the wild type plants lost more water during drought-stress. This could possibly mean that plants with bigger leaf area lose more water during drought-stress conditions compared to those with smaller leaf areas. According to a study done by Slabbert and van den Heever (2007) on 33 amaranth germplasm collected from different communities in South Africa, they found that drought-tolerant plants were able to maintain bigger leaf areas. While Blum (1997) concluded that a decrease in leaf area due to drought-stress is considered an avoidance mechanism that allows less water loss through transpiration. We could speculate that the mutant lines shut down transpiration through wilting and as a result they recovered easily after re-watering.

The mechanisms that allow the production of crops under unfavourable conditions can either be a result of drought-tolerance in the event of cellular dehydration, or the ability to adjust water loss so that the water status can be maintained for leaf development (Liu and Stützel, 2002). Generally, the number of leaves per plant is reduced significantly by drought conditions. During drought-stress, it has been shown that physiological alteration that occurs due to severe water deficit, leads to the development of smaller and compact cells and bigger specific leaf weight (Chung *et al.*, 1997). In this study, the two mutant lines developed less leaves than the wild type as a mechanism to adjust to

drought conditions. The wild type had more leaves under both well-watered and drought-stress conditions compared to the two mutant lines, but failed to survive the drought treatment. The number of leaves per plant was also reduced in groundnut (*Arachis hypogea*) under drought-stress (Reddy, *et al.*, 2003).

In sunflower plants, drought-stress adversely affected the overall biomass production compared to the well-watered plants (Manivannan *et al.*, 2007. Drought-stress conditions also decreased the fresh weight (FW) of the whole plant of all the *Amaranthus* lines. A decrease in the whole plant's FW under water deficit might be due to suppression of cell growth due to low turgor pressure. Similar observations were also found in wheat (Rane *et al.*, 2001) and pearl millet (Kusaka *et al.*, 2005).

Anthocyanin has been shown to improve drought-tolerance in some plants, since anthocyanin is involved in contributing to osmotic adjustment during drought-stress and low temperature (Chalker-Scott, 1999). The pink colour of the stem stalks in both mutant lines was due to a higher anthocyanin content which might contribute to improved drought-tolerance of these lines (Slabbert *et al.*, 2004). In this study, anthocyanin content differed significantly between the mutant and the wild type lines. The stems stalks of the mutant plants contained more anthocyanin than the wild type under well-watered conditions. It may be possible that anthocyanin plays a role in the reaction of the mutant lines to drought-stress, and thus contributed to the recovery of these mutant lines after water deficiency. These results are similar to a study done by Efeoğlu *et al.* (2009) on the physiological responses of three maize cultivars (Doge, Vero and Luce) to drought-stress and recovery. They found that plants with more anthocyanin under well-watered conditions had lower levels of anthocyanin during drought and recovered better after re-watering.

The main factor resulting in reduced growth in response to drought is leaf RWC (Morgan, 1992). RWC can be used to determine the relative water status in plants during

drought-stress conditions, indicating the ability of the plant to overcome severe drought conditions (Morgan, 1992). In the initial stage of leaf development, RWC of the leaves is higher and decreases as the dry matter accumulates and the leaf matures (Reddy *et al.*, 2003). In our study, the RWC of the leaves of all drought-stressed lines decreased with time as compared to the well-watered plants. Due to the decrease in soil water, there was a shortage in water supply to the plants. As a result, the RWC decreased in all genotypes. The wild type plants showed a greater decrease in RWC as compared to mutant #2 and mutant #5 after 19 days of drought-stress, but the difference was not significant statistically. As a result, no wild type plants recovered after re-watering, but the two mutant lines recovered after re-irrigation. These results are in contrast with a study done by Liu and Stützel (2002) who found that all the genotypes used during their study, including *A. tricolor*, recovered after seven days of re-watering. Slabbert and van den Heever (2007) measured RWC from 33 amaranth germplasm including *A. tricolor*. They showed that *A. tricolor* was one of the species that was able to recover after a drought period of 14 days when re-watered. Also, in a study by Omami and Hammes (2006) on *A. tricolor* and *A. cruestus*, the effects of polyethylene glycol (PEG)-mediated drought-stress on the growth of the plants and leaf water relations showed that, at the end of the water deficit period, the RWC of the *A. tricolor* plants was reduced from 86 percent to 58 percent. The RWC of *A. cruentus* decreased from 87 percent to 60 percent. They found that after re-watering, both genotypes returned back to the original RWC levels.

The low soil matric potential associated with reduced water content causes a decrease in water potential in plants resulting in reduced rate of plant growth (Omami and Hammes, 2006). In this study, there was a decrease in the mass of the pot weights for all the *Amaranthus* lines exposed to drought treatment, thus indicating a decrease in plant mass.

When the plants were re-watered after 19 days of drought-stress conditions, full recovery in the mutant lines were observed after seven days of re-watering. In a study

done by Slabbert and van den Heever (2007) on 33 amaranth germplasm, 14 day drought-stressed plants were able to recover 15 hours after re-watering. The longer the drought treatment, the longer it took for the plants to recover.

5.2. Biochemical studies

The presence of low water in the soil results in less plant growth which leads to protein accumulation in plants (Showler, 2003). The effect of drought-stress on plants can either be positive or negative, depending on the duration of stress, growth stage of the plant and the intensity of drought (Sharma *et al.,* 2004). In this study, protein content was determined using the leaves of the wild type and two mutant lines during both drought-stress and well-watered conditions. Under drought-stress conditions, the protein level from all the lines were affected and mutant #2 had a higher protein content as compared to mutant #5 and the wild type line. A decline in protein content was pronounced after 13 days of drought-stress in all the lines. In our study, under control conditions, all the *Amaranthus* lines had an increase in protein content over time with mutant #2 exhibiting higher protein content compared to mutant #5 and the wild type. By contrast, during a study by Manivannan *et al.* (2007) on sunflower (*Helianthus annuus* L.) varieties, drought-stress caused an increase in amino acid content compared to the well-watered plants. An increase in amino acid content during drought-stress was also observed in sorghum (Yadav *et al.*, 2005). The increase in amino acid content may be the result of protein hydrolysis, as well as osmotic adjustment of the cellular contents (Manivannan *et al.*, 2007). During a study by Salekdeh *et al.* (2002), two rice cultivars (*Oryza sativa* L. cv CT9993 and cv IR62266) were analysed to investigate the changes in the leaf protein during drought-stress and after recovery, using proteomic analysis. According to their findings, leaf proteins were reversible in both the shallow-rooted drought-tolerant (*Oryza sativa* L. cv IR62266) cultivar and the deep-rooted drought-avoidance (*Oryza sativa* L. cv CT9993) cultivar. They concluded that the increase in protein concentrations may be due to changes in protein synthesis. Decreases may be due to

breakdown or due to binding of the proteins to cellular components. In our study other proteins might have started to degrade after 13 days of drought-stress conditions or biosynthesis stopped due to drought-stress conditions.

5.3. Genomic studies

Random amplified polymorphic DNA (RAPD) can be used to determine the DNA variation among the mutants and non-irradiated wild type lines. Generally, the presence or absence of RAPD bands can either show similarities and/or indicate diversity between different species (van der Vyver, personal communication). During this study, 19 arbitrary primers were used to screen the wild type and the two mutant lines. Two primers (OPA-07 and OPA-16) produced visible and different RAPD profiles. Thus, it was possible to detect differences between the mutated and non-mutated lines using RAPD analysis. These two primers can be used for screening larger populations in order to determine if they can be used as markers for drought-tolerance. During a study done by Ray and Roy (2009), they analysed the genetic diversity and phylogenetic relationships among six *Amaranthus* species (*A. gangeticus* (syn. *tricolor*), *A. paniculatus*, *A. viridis*, *A. hypochondriacus*, *A. caudatus* and *A. cruentus*) from different phytogeographic regions using RAPD markers. They found that polymorphisms were detected between all six species using primer OPA-20 and *A. tricolor* sustained greater genetic variation compared to the other *Amaranthus* species using primer OPA-20.

CHAPTER 6

6. Conclusion and Recommendations

6.1. Conclusion

The aim of this study was to characterise drought-tolerant *A. tricolor* mutant lines produced by radiation. The objectives were to investigate the performance of these lines, including the wild type, under both well-watered and drought-stress conditions. Furthermore, the genetic diversity was analysed in all the lines using the RAPD technique.

Mutagenic treatment used to produce mutant lines may directly affect cell division and some intrinsic growth characteristics, which result in delaying the rate of plant development (Beggs *et al.*, 1985). This may cause changes in the plant height, total biomass, number of leaves and leaf area. Radiation dwarfed mutant #2, but greatly increased the shoot height of mutant #5 compared to its wild type.

The results presented in this study showed the behaviour of well-watered *A. tricolor* plants in comparison with the drought-stressed plants. The wild type plants had more leaves and larger leaf area. As a result, they lost more water during drought conditions compared to the two mutant lines, which retained more water under drought conditions. The two mutant lines exhibited higher drought-tolerance level than the wild type line in terms of recovery after 19 days of drought-stress conditions. Both mutant lines also showed an increase in protein content per gram dry weight under well-watered and drought-stress conditions. However, water deficit affected protein concentration in all the lines after 13 days of drought-stress. Under drought-stress conditions, mutant #2 performed better when compared to mutant #5 and the wild type by having higher protein content in the leaves and an increased survival of plants after drought treatment.

Therefore, mutant #2 would be a good candidate for further experiments on *Amaranthus* cultivation under extreme drought conditions. Molecular techniques, specifically RAPD, detected polymorphisms between the wild type and the two mutant lines. Possible markers were identified in both mutant lines. From this study it can be concluded that radiation improved drought-tolerance in the mutant lines.

6.2. Recommendations

- More genetic studies still need to be done in order to identify how genes changed in their expression due to radiation.
- Primer OPA-20 should be used to detect polymorphism between the two mutant lines and the wild type.
- Genes that are differentially expressed between the wild type and mutant lines, and are responsible for drought-tolerance in the mutant lines still need to be isolated and characterised further by using bioinformatics tools.
- Techniques such as cDNA amplified fragment length polymorphism (cDNA-AFLP) and cDNA representational difference analysis (cDNA RDA) can be incorporated and used to detect genetic variations.

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