EVALUATION OF TUBERS FROM CASSAVA TRANSGENIC LINES TOLERANT TO *AFRICAN CASSAVA MOSAIC VIRUS*

by

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Dissertation

Submitted in fulfilment of the requirements for the degree

Master of Science

in

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

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June 2020

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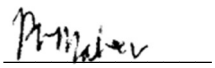
Signature:  08/09/2020

Abstract

Cassava (*Manihot esculenta* Crantz) is a crop plant that produces starchy tuberous roots. The cassava plant is resilient to abiotic stress and very efficient at producing carbohydrates, making it a valuable crop in the production of starch, for industrial uses. Some of the main causes of yield loss in cassava plants are viral diseases, such as cassava mosaic diseases (CMD), caused by cassava mosaic geminiviruses (CMG). One of the most promising means to combat CMGs is the development of transgenic crops. AMM2-52, CMM6-2 and CMM6-6 transgenic lines were developed previously and have been proven tolerant to *African cassava mosaic virus* (ACMV), a CMG. After transgenic cassava lines are produced, it is necessary to perform tests to evaluate the potential effects of the transgenes on the tubers of the plant. There are many potential sources of variation in the process of developing transgenic plants, that can result in altered tuber production or composition. For preliminary studies, before field trials are performed, cassava is grown in highly controlled laboratory environments with sub-optimal conditions (such as soil, lighting, temperature, and water availability) for tuber production. Consequently, the transgenic ACMV tolerant cassava plants in this study were grown under non-field, controlled, growth room conditions, with pot size and salicylic acid (SA) foliar spray sub-treatments, that could potentially improve tuber production and growth. The growth characteristics and resulting tubers were also analysed, and modelled with nonlinear models, in comparison with the dsAC1 line (proven resistant to ACMV), cv.60444 cultivar (model cultivar that the transgenic lines are transformed from) and TME3 (CMD tolerant) landrace. At the end of the study, the three ACMV tolerant transgenic lines did not display any noticeable irregularities in starch composition and growth characteristics. The starch content of the tubers was justifiably low, due to the young age (six months) of the plants when the tubers were harvested, where smaller tubers correlated with a lower starch content. Amylose content of the tubers was below 30% for the most part, as is expected in cassava tubers. Starch granule morphology and size was uniform among the transgenic lines and control landraces tested. A method to reliably produce tubers in indoor growth facilities was not successful, but it was found that bigger pots resulted in a greater chance of tubers being produced and often resulted in an increased growth rate in the cassava plants. The salicylic acid treatment had no strong effect on tuber production, or growth of any of the cassava plants. Early growth, based on the height of cassava, in controlled environments, was successfully modelled, with the logistic model having the best fit. Leaf number was found to

be unsuitable for nonlinear modelling, and leaf area index (LAI) or yield of leaves should be used instead.

Acknowledgements

- Prof. Chrissie Rey, for running one of the few labs in South Africa with transgenic plant research, allowing me to work with said transgenics, and providing me support for my research throughout.
- All of my lab mates who provided indispensable help, engaging discussion, and heartening banter.
- My family, for their stalwart support, and always encouraging me to pursue my ambitions.
- University of the Witwatersrand for the Postgraduate merit award.

Lists of abbreviations

ACMV [NG:Ogo:90] : *African cassava mosaic virus* [Nigeria:Ogorocco;1990]

AIC : Akaike information criterion

ANOVA : Analysis of variance

ASA : Acetylsalicylic acid

BIC : Bayesian information criterion

CBSD : Cassava brown streak disease

CMD : Cassava mosaic disease

CMG : Cassava mosaic geminivirus

cv. : Cultivar

DAP: : Days after potting

DNA : Deoxyribonucleic acid

DIC : Differential interference contrast

dpi : Days post infection

ds- : Double stranded

FEC : Friable embryonic callus

GM : Genetically modified

GOPOD : Glucose oxidase plus peroxidase

HI : Harvest index

IR : Inverted repeat

LAI : Leaf area index

miRNA : Micro RNA

mRNA : messenger RNA

MS20 : Murashige and Skoog medium with 20g/L sucrose

NPK grade	: Nitrogen, Phosphorous, Potassium ratio
NSP	: Nuclear shuttle protein
PCR	: Polymerase chain reaction
PTGS	: Post-transcriptional gene silencing
qPCR	: Quantitative Polymerase chain reaction
RdRp	: RNA-dependent RNA polymerase
Rep	: Replication initiator protein
Ren	: Replication enhancer protein
RISC	: RNA-induced transcriptional silencing
RMSE	: Root mean square error
RNA	: Ribonucleic acid
RNAi	: RNA interference
ROS	: Reactive oxygen species
RSS	: Residual sum of squares
SA	: Salicylic acid
SACMV	: *South African cassava mosaic virus*
siRNA	: Small interfering RNA
ss-	: Single stranded
T-DNA	: Transfer Deoxyribonucleic acid
Ti plasmid	: Tumour inducing plasmid
TrAP	: Transcriptional activator protein
VSR	: RNA-silencing suppressor

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Chapter 1: Literature review

1.1. Cassava

Cassava (*Manihot esculenta* Crantz) is a woody perennial shrub with starchy tuberous roots (Ihemere et al., 2006; Kuria et al., 2017). Cassava is an outbreeding species, and is considered an amphidiploid, with 2n = 36 chromosomes (El-Sharkawy, 2003). While its exact geographical location of origin is not under scientific consensus, recent molecular evidence, such as phylogenetic analysis, places it as being domesticated on the southwestern rim of the Amazon basin (Lebot, 2009; Léotard et al., 2009). The same molecular evidence also suggests that modern cassava was derived from *M. esculenta ssp. flabellifolia*, its closest wild relative.

Cassava is mainly cultivated for its root tubers, which are a valuable source of dietary carbohydrates. The leaves and shoots are also consumed in some regions and contain high quality proteins and fats. Because of the perennial nature of the plant, and the fact that they need to be consumed fresh, these can only be harvested in certain seasons (Balagopalan, 1988). Cassava crops are usually vegetatively propagated by mature woody stem cuttings, and the tubers are often harvested 7-24 months after planting (El-Sharkawy, 2003). Cassava roots and leaves release cyanogenic glucosides, sometimes in quantities great enough to be toxic to humans (Lebot, 2009). To allow for human consumption, cultivars with a lower cyanide content are cultivated, and harvested cassava roots and leaves are processed in a manner that removes the cyanide. The cassava roots are processed into a variety of forms before consumption, such as cassava flour and bread, which prolong the longevity of the roots; that usually deteriorate a few days after harvesting.

Cassava is grown in tropical and sub-tropical regions, and is produced and consumed primarily in Africa, Latin America and Asia; where it is also known as manioc, tapioca and yuca (Legg et al., 2015). The cassava plant is very efficient at producing carbohydrates, which are highly sought after in the tropical environments that they are grown in (Izumi et al., 1999). The largest producer of cassava tubers is Nigeria, which produced an average of approximately 48.3 million tonnes of cassava per year from 2007 to 2016 (FAOSTAT, 2017). Thailand and Brazil follow with around 28 million tonnes and 24 million tonnes produced respectively over the same period. Cassava is also grown in South Africa, though at a much smaller quantity, and usually by subsistence farmers rather than commercially.

1.2. Cassava growth characteristics

There is a large variety in morphology and growth of various cultivars of cassava, though some characteristics are shared between most cultivars (***figure 1.1)***. The main components of all cassava plants are the nodal units, consisting of a lamina (leaf blade), a long petiole, and internode; and roots, which may develop into tuberous storage roots. Cassava is categorised as having indeterminate growth, with internodes of varied length and mass depending on the cultivar, age, and environmental factors (El-Sharkawy, 2003). Most cassava cultivars usually grow to a maximum of between 1-4 m in height. Like all other species of the *Manihot* genus, cassava has well defined lenticels and vascular tissue (Lebot, 2009). Cassava plants generally show strong apical dominance and rarely produce leaves from the axillary buds (Cock et al., 1979). The base of each petiole of mature leaves is usually surrounded by two stipules. (Alves, 2002). The lamina of a mature leaf is glabrous, has palmate veins and is lobed, with between three to eleven lobes, depending on genotype and leaf size. There are between 278 to 700 stomata per mm^2, mostly situated on the underside of the leaf, with paracytic morphology (Alves, 2002; El-Sharkawy & Cock, 1987). There is ample evidence suggesting that cassava employs the C_3 mode of photosynthesis (Obata et al., 2020). Around four months after planting, cassava in the field forms a deep (>2m), sparse fine-root system allowing lower water reach during dry periods (Duque & Setter, 2013; El-Sharkawy, 2003).

Figure 1.1 **Various cassava cultivars and lines, grown in a greenhouse**

If planted from seeds, a taproot system develops, while plants from stem cuttings form an adventitious root system from the base of the cutting (Alves, 2002; ***figure 1.2***). The taproot and some adventitious roots will later enlarge to form root tubers. Seed grown cassava plants,

which are generally only used in breeding programs, are less vigorous, and take a longer time to sprout, compared to plants grown from stem cuttings (Alves, 2002). In the field, stem cuttings utilise reserves in the stem to start growing, and usually start to sprout and root one week after planting in favourable conditions (Howeler et al., 2013). Upon sprouting, one or more axillary buds on a cutting develop rapidly and form the nodal units. The plant generally develops shoots and a fine root system during the first two months (El-Sharkawy, 2003). Leaf growth generally occurs from the top of the plant in a spiral pattern around the stem, at varying rates. Abscission of leaves is acropetal (from the bottom of the plant, up), and results in visible scars. Leaf formation per apex decreases with age (Veltkamp, 1985). The total number of leaves produced per plant depends on the environment, genotype and number of branches (El-Sharkawy, 2003).

Figure 1.2 **A) Adventitious root system of cassava growing in a small pot, with tuber formation on the bottom right B) Adventitious root growth of cassava, as viewed in a transparent plastic box**

Irikura et al. (1979) describe two basic types of branching (also called forking) that occur in cassava. Axillary buds on the lower part of the stem develop into branches, in one type. In the other, when apical dominance ceases, the main apex becomes reproductive and two or more axillary buds immediately below the main apex develop into approximately equally sized branches. There can be between one to four branches per point. Branching behaviour is highly variable among cultivars, in terms of timing, number of branches at each point and height of the first branching point (Lian & Cock, 1979). The branching habits of the plant will determine the number of active apices it has.

Leaf area index (LAI) is the ratio of leaf area per unit of ground, that describes the rate of leaf formation per apex, apex number per unit area, leaf size, and leaf life of a plant (CIAT, 1976-2008). Crop growth rate in most crops increases as LAI increases up to a point, then remains constant or declines, even if the LAI continues to increase. Cock et al. (1979) found a maximum cassava tuber growth rate at an LAI of 3.5 to 4.0, followed by a sharp decline of tuber growth rate in the MCol 113 cultivar as its LAI exceeded 4.0.

1.2.1. Environmental conditions affecting cassava growth

Once mature, cassava plants are quite hardy, and are resilient to most abiotic stresses (El-Sharkawy, 2003). Cassava has been cultivated between latitudes of 30˚N and 30˚S, with annual precipitations ranging from 500 mm up to 6000-8000 mm and soil pH between 4.5 and 8 (El-Sharkawy et al., 1992). Cassava has also been recorded being cultivated in the high-altitude tropics (up to 2300 m above sea level), with high yields (El-Sharkawy et al., 1992).

Most cassava cultivars, being tropical plants, require a warm climate, averaging more than 20°C for greater growth and leaf photosynthesis (with an optimum leaf temperature of 25°C-35°C (Irikura et al., 1979)). These temperatures (>20°C) have also been recorded as causing branching to occur earlier in cassava (Irikura et al., 1979). Keating and Evenson (1979) found that field planted cassava cuttings could sprout from 12-40°C, with optimal sprouting occurring between 28.5°C-30°C, after testing the MAus 7 and MAus 10 region-adapted cultivars. These sprouting temperatures might not be representative of cassava plants in general, though no other, comprehensive studies on this trait in cassava have been recorded. Higher temperatures are also associated with a greater LAI in cassava, while lower temperatures have the effect of increasing leaf survival (Alves, 2002). At high temperatures, a leaf is fully expanded in two weeks and the size increases with plant age up to about four months and then declines; however, at low temperatures the maximum size decreases and is achieved at late growth stages (Irikura et al., 1979)

Mature cassava plants are highly drought tolerant, which can in part be attributed to the sensitivity of their stomata to atmospheric and edaphic water stress (El-Sharkawy, 1993; El-Sharkawy et al., 1985). Cassava stomata partially close in low air humidity and in response to poor soil, and water shortage, mitigating dehydration and keeping the leaf photosynthetically active (CIAT, 1976-2008; Duque & Setter, 2013; El-Sharkawy, 1993, 2003; El-Sharkawy et al., 1985). The deep fine-root system also aids the plant during dry periods, by absorbing water from deeper strata of the soil (Duque & Setter, 2013). In dry conditions, cassava will also deplete starch and sucrose reserves from leaves, after which, leaf abscission occurs (Duque & Setter, 2013; El-Sharkawy, 2003). This benefits the plant by reducing transpiration water loss that occurs in the leaf canopy. To add to this effect, the rate of leaf formation also slows down and leaves are smaller, with greater longevity, during dry periods (Connor et al., 1981; Irikura et al., 1979).

The optimal light period is 12 hours (with high solar radiation), and optimal temperature is between 30 to 40°C for photosynthesis in cassava (Berry & Bjorkman, 1980; De Souza et al., 2017; El-Sharkawy et al., 1992). Photosynthetic rate may decrease in plants with mechanical injuries and shock, due to an increased production of ethylene (El-Sharkawy et al., 1992). Small pots may also lower rates of photosynthesis, by inhibiting root growth, thereby lowering the capacity to store carbohydrates (El-Sharkawy et al., 1992). Cassava grown in greenhouses or growth chambers sometimes have thin leaves, with a lower mass and lower activities of photosynthetic enzymes due to exposure to suboptimal lighting levels (Berry & Bjorkman, 1980; El-Sharkawy et al., 1992). However, the variability of the plants grown in these controlled conditions is considerably smaller than in the field (Mahon et al., 1976).

1.2.2. Cassava tuber production

Figure 1.3 **Cassava tubers sold at a market (Monniaux, 2005)**

In cassava, starch deposition in roots has been observed around 25–40 days after planting, in many cultivars (Cock, 1984). The reason this process is initiated could be because the photo-assimilates of the plant are greater than needed for shoot growth (Cock et al., 1979). Typically, some secondary growth begins in some of the fibrous roots after three months, forming micro-tubers (Alves, 2002). These swell with starch obtained by the leaf canopy, and are well developed root tubers by the sixth month, when the plant achieves maximum canopy size (Howeler et al., 2013). The tuberous roots have a reduced capacity to absorb nutrients and water from the soil compared to their precursor fibrous roots (Alves, 2002). Izumi et al. (1999) found that the number of non-storage roots decreased with the initiation of tuber bulking. The tubers consist of a barky periderm, cortex, starchy flesh layer (parenchyma) and central vascular bundle, as seen in ***figure 1.3*** and ***1.4*** (Alves, 2002;

Onwueme, 1978). The tubers can serve as a source of carbohydrates and water for growth, metabolism, respiration and osmolyte synthesis during dry periods (Duque & Setter, 2013).

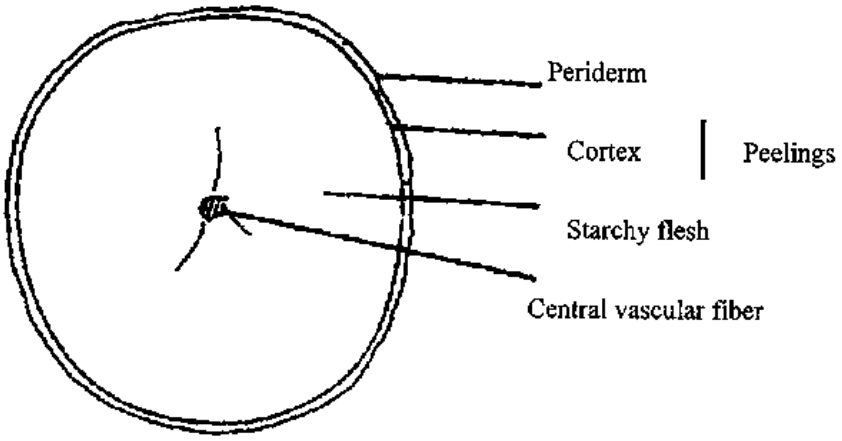

Figure 1.4 **Diagram of a cross section of a cassava tuber (Onwueme, 1978)**

In the tropics, cassava has been shown to have high yields of tuber growth, when grown in optimal conditions, such as those found in the Patia Valley in Colombia (CIAT, 1976-2008; El-Sharkawy, 2003). However, in many small farms and in laboratory conditions, these high yields are difficult to replicate. Traditional methods of improving crop yield, such as high fertilisation and irrigation do not result in consistently high tuber yields for cassava. In many cases, the optimal conditions for tuber growth are sub-optimal for the growth of the plant (CIAT, 1976-2008). In addition, other characteristics of the tubers, such as starch content and morphology, vary between cultivars.

The typical composition of cassava tubers is: 70% water, 24% starch, 2% fibre, 1% protein, with the remainder being other substances, including minerals (Tonukari, 2004). Apart from water, starch is the primary component, and dry starch yields have been found between 73.7% and 84.9% of the tuber (Baguma, 2004). Starch is a polysaccharide of glucose, with two polymeric structures: amylose and amylopectin. Amylopectin is the major polymer, constituting 70% or more of the cassava starch, while amylose is the minor, usually constituting 30% or less (Taggart & Mitchell, 2009; Wang et al., 2018). In various cultivars, Defloor et al. (1998) found amylose contents of the starch samples varied between 17.9% and 23.6%. Starch is accumulated as granules in the amyloplasts of the plant's storage organ cells (Keeling & Myers, 2010; Wang et al., 2018). Cassava starch granules are rounded, oval, or truncated in form with a characteristic birefringence, and sizes ranging from 5 to 40 μm (Ceballos et al., 2007; Vasconcelos et al., 2017).

Cassava can produce tubers in low fertility soil, but at lower yields. In over-fertilised soil, the plant will sometimes allocate more resources to stem growth, to the detriment of tuber production (Fermont et al., 2009; Pellet & El-Sharkawy, 1993). Soil compaction can result in reduced cassava tuber yields in young plants, while increasing LAI (Maduakor, 1993). Cassava tubers can last for more than a year below the soil, but rapidly deteriorate after being harvested, and need to be processed within two days (Koehorst-van Putten et al., 2012; Reilly et al., 2003).

Mahon et al. (1976) found that in controlled environments, with mature plants, the proportion of dry matter in the roots increased in low-temperature plants at a greater rate than plants kept at high temperatures. This might be explained by the increased leaf growth and increased shading of the lower leaves at higher temperatures, which would decrease the mean net photosynthetic assimilation rate to the tubers. Most tuber producing crops, including cassava, show a yield reduction in conditions of water deficit, with cassava losing around 30% of its yield, and producing fewer tubers (Daryanto et al., 2017; Vandegeer et al., 2013). Dry conditions have a more detrimental effect on lateral root development than on the taproots or main roots in cassava (Duque & Setter, 2013). Cassava tubers are most vulnerable to drought damage between one to five months after planting (Connor et al., 1981). When carbohydrate production is too low for the cassava plant, the carbohydrates from the tubers are utilised until the plant dies from carbohydrate starvation, or other causes (Duque & Setter, 2013). The total biomass and storage dry root yield of cassava correlates with the mean seasonal upper canopy leaf photosynthetic rate (El-Sharkawy & Cock, 1990; El-Sharkawy et al., 1990). Root growth has been linked to LAI in cassava, where cultivar Col 113 showed a sharp decrease in root growth rate at LAIs greater than four (Cock et al., 1979). This could be because the LAI increases approximately linearly with the resources the leaves require, and will eventually tap into the tuber starch reserves (CIAT, 1976-2008; Cock et al., 1979).

1.3. Cassava mosaic geminiviruses

Viral diseases, such as cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) are a major cause of cassava yield losses in tropical Africa and Asia (Legg et al., 2011). CMD is the most severe and widespread, and leads to mosaic, mottling, misshapen and twisted leaflets, an overall reduction in size of leaves and plants, and reduced or absent tuber production (Alabi et al., 2011; ***figure 1.5***). Cassava mosaic disease is caused by cassava mosaic geminiviruses (CMGs). CMGs belong to the family *Geminiviridae*, genus

Begomovirus, which are circular single-stranded DNA (ssDNA) viruses that infect crops throughout the tropical and subtropical regions of the world (Fauquet et al., 2008; Rey & Vanderschuren, 2017). *Geminiviridae* are characterized by the vectors that transmit them, with begomoviruses being transmitted by the whitefly, *Bemisia tabaci* Genn. (Berry et al., 2004; Legg et al., 2014a). *Begomovirus* is the largest genus of viruses with respect to the number of species included (Brown et al., 2015); containing 388 species according to the International Committee on Taxonomy of Viruses (ICTV; Adams et al., 2017). This genus contains all CMGs, with species such as *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* and *South African cassava mosaic virus* (SACMV), which are widespread in southern Africa (Rey et al., 2012). The recent spread of geminivirus-associated diseases has led to large economic losses, and in the case of ACMV (and related viruses), the annual losses are about US$1.9–2.7 billion, with the cassava disease pandemic in East and Central Africa causing severe hardship and problems in the regions (Scholthof et al., 2011). The emergence of new virus strains and the increasing number of virus carrying whitefly vectors, possibly due to insecticide resistance and climate change, has increased the spread of CMGs in cassava crops (Seal et al., 2006). Due to the nature of cassava propagation, typically through stem cuttings, cassava is highly vulnerable to CMGs which are transmitted in this manner (Beyene et al., 2016; Legg et al., 2015).

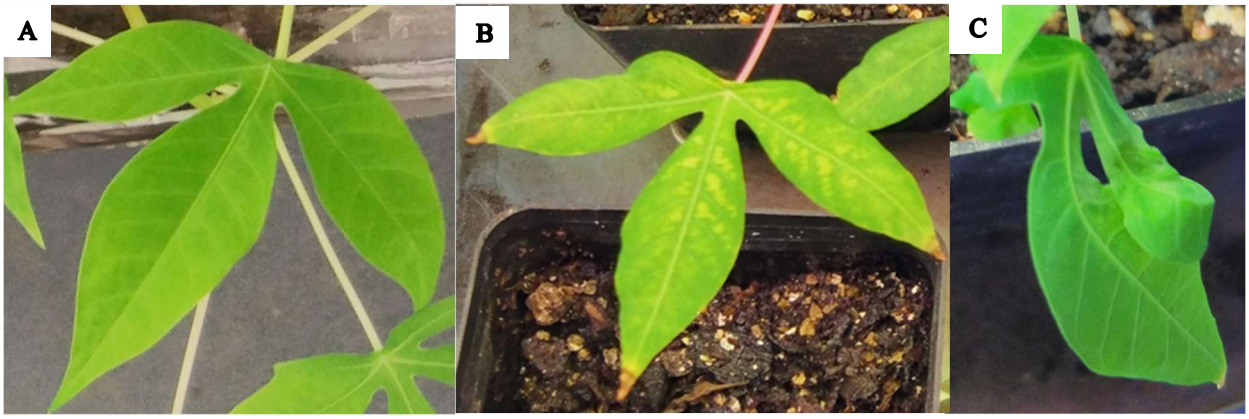

Figure 1.5 **Cassava cv.60444 leaves at 28dpi, A) Healthy leaf from mock-inoculated plant, B) Chlorosis in leaf from ACMV-infected plant, C) Curling deformities in leaf from ACMV-infected plant**

CMGs possess a bipartite ssDNA genome, comprising of covalently closed, circular DNA-A and DNA-B components, which are encapsidated by a 30×20 nm twinned icosahedral particle (Rey & Vanderschuren, 2017). The DNA-A and DNA-B molecules are each around 2.7–2.8 kilobases in size, and share a common region of 200 nucleotides (***figure 1.6***) (Brown et al., 2015; Rey & Vanderschuren, 2017). The origin of replication for DNA-A and B are found in

the common region. There are six open reading frames (ORFs) on DNA-A, each encoding a specific protein, namely: AC1, the replication initiation protein (Rep); AC2, the transcriptional activator protein (TrAP); AC3, the replication enhancer protein (Ren); AC4, the viral RNA-silencing suppressor (VSR); AV1, the coat protein; and AV2, putative protein kinase. DNA-B has two ORFs: BV1, which encodes the nuclear-shuttle protein and BC1, which encodes the movement protein (Legg et al., 2015). Once entering a host, by means of whitefly transmission, a CMG begins a cycle of DNA replication, DNA accumulation and virus assembly, as well as virus spread in the host. The viruses replicate by means of a rolling circle amplification through a dsDNA intermediary stage (Hanley-Bowdoin et al., 1999).

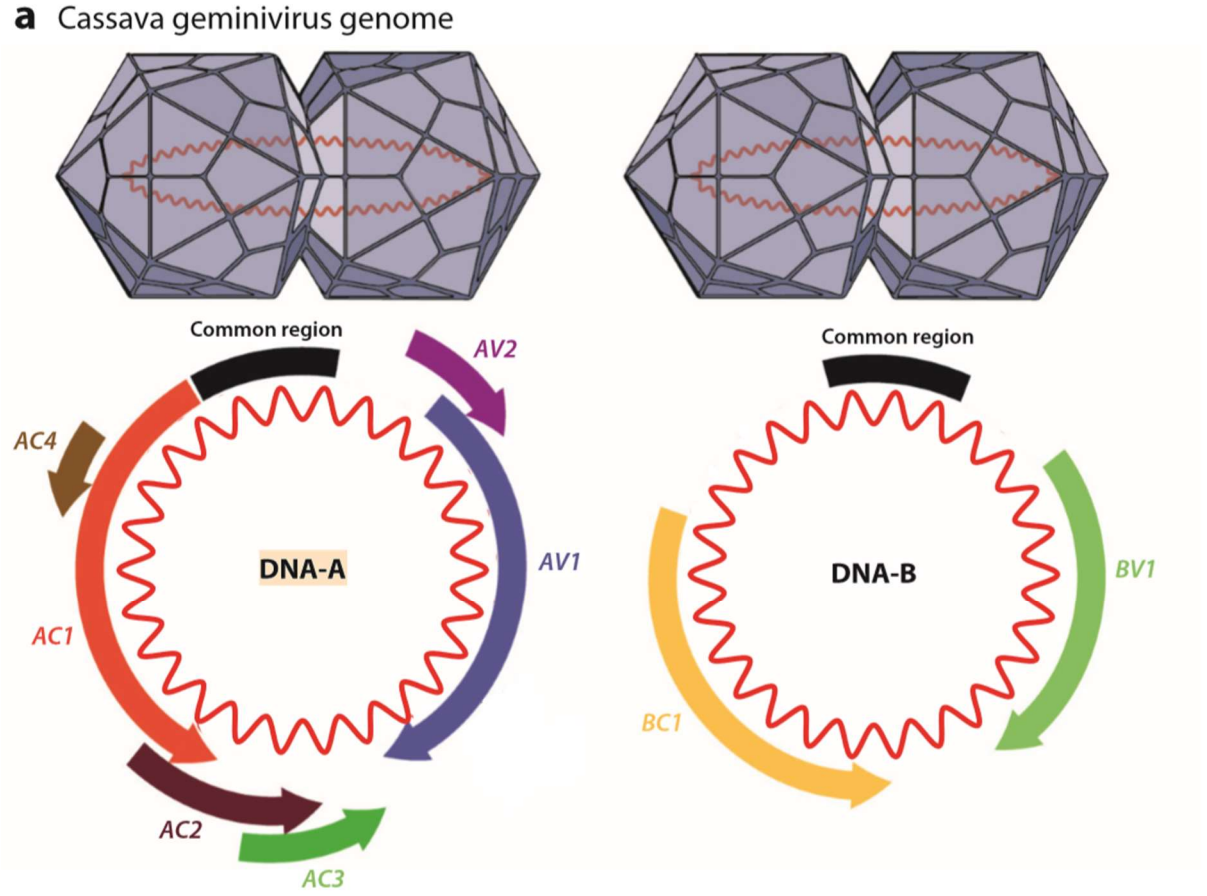

Figure 1.6 **Structure of a CMG capsid and DNA-A and B components**

DNA-A: AV2 (encodes putative protein kinase), AV1 (encodes coat protein), common region, AC1 (encodes replication initiation protein), AC2 (encodes transcription activator protein), AC3 (encodes replication enhancer protein), AC4 (encodes silencing suppressor protein). DNA-B: BV1 (encodes nuclear-shuttle protein), common region, BC1 (encodes movement protein). (Rey & Vanderschuren, 2017)

1.4. Genetic modification of cassava

In the wild, cassava has a high tendency to outcross, which results in a high heterozygosity (Lebot, 2009). In addition to its high heterozygosity, there is poor flowering and a strong inbreeding depression in farmer selected landraces, making cassava unamenable to conventional breeding (Legg et al., 2015). Tolerant landraces and cultivars exist, but using these to breed for CMD resistance is especially difficult due to the polygenic character of the CMD resistance trait and the rapid evolution of geminiviruses (Zhang et al., 2005). While cassava remains recalcitrant to most conventional genetic engineering strategies, mostly because of difficulties in genetic transformation, new adapted methods have assisted to improve its recalcitrance. (Taylor et al., 2004). Recent advances have made it possible to create transgenic cassava, with agrobacterium-mediated transformation being one of the more commonly used methods (Chetty et al., 2012; Msikita et al., 2006).

Before transformation of cassava, there needs to be production of embryogenic tissues from in vitro explants, such as friable embryonic callus (FEC) (Bull et al., 2009; Taylor et al., 2004). *Agrobacterium tumefaciens* is used to introduce transgenes directly into FEC using recombinant vectors (Bull et al., 2009; Taylor et al., 2004). Other methods of cassava modification include the particle bombardment method, and more recently, using the CRISPR/cas9 system (Odipio et al., 2017). Genetic modification is being researched to increase the starch yield of cassava tubers, as well as for biofortification of protein and vitamins (Adenle et al., 2012; Ihemere et al., 2006; Vanderschuren et al., 2013). However, the major focus is on engineering disease resistance, due to the large yield losses where cassava is grown. The most promising direction to achieve this end, is the use of RNA silencing techniques to engineer cassava to express siRNAs targeting the viruses (Rey et al., 2015).

1.4.1. Effect of *Agrobacterium*-mediated transformation

Wild, unmodified *Agrobacterium tumefaciens* causes crown gall disease by transferring a fragment of its Ti (tumour-inducing) plasmid, called T-DNA (transfer DNA), into plant cells (Chilton et al., 1977; Godwin et al., 1991). This process has been altered, where foreign DNA is integrated into the T-DNA. This prevents the gall disease causing elements, and allows for the integration of transgenes into the plant cells (Barton et al., 1983). Agrobacterium-mediated transformation has a few key advantages over other methods of plant genetic engineering, such as allowing the transfer of relatively large segments of DNA, the

integration of small numbers of copies of genes into plant chromosomes and minimal rearrangement of transferred DNA (Komari & Kubo, 1999).

In practice however, there are many complications that can occur in transgenic plants created by agrobacterium-mediated transformation, and the stable and predictable expression of transgenes remains problematic (Gelvin, 2003). There is sometimes the formation of repeats of transgenes, certain rearrangements and integration of non-target DNA segments (Komari et al., 2004). The position that the T-DNA, with the transgene, is integrated into the plant genome is variable. This means that the T-DNA could integrate at unpredictable distances from transcriptional activating elements or enhancers, affecting the expression of the transgenes they carry (Gelvin, 2003). Multiple copies of a transgene in a plant genome often occurs when transgenic plants are generated by direct DNA transfer methods, such as using polyethylene glycol or particle bombardment (Gelvin, 2003). These copies can be in tandem or inverted repeat (IR) arrays, in either multiple or single loci. This can often result in transgene silencing from transcriptional mechanisms, usually associated with methylation of the transgene promoter (Meyer, 2000). In addition, posttranscriptional silencing can occur resulting in unstable transcribed RNA (Gelvin, 2003).

Phenolic compounds, such as acetosyringone (released by wounded tobacco plant cells) and media with a low pH (<5.7) are often used in agrobacterium-mediated transformation protocols, including cassava FEC transformation (Bull et al., 2009). Antibiotics, such as kanamycin and carbenicillin are also utilised to eliminate the agrobacterium cultures, but these are slightly toxic to some plants (Ling et al., 1998). These treatments, including *in vitro* conditions for transformation often stress the plants, and may lead to somaclonal variation (Komari et al., 2004).

Schreuder et al. (2001) found that with cassava, even when FEC lines with a good overall performance are used, variation within and between experiments occurs. More recently, Taylor et al. (2012a) reported modified agrobacterium-mediated transformation methods of selected cassava cultivars were robust and express traits well under greenhouse conditions. Cassava FEC transformation by agrobacterium is constantly being improved, such that it is more reliable, and can transform elite landraces that farmers prefer; which are often recalcitrant to this method of transformation (Chauhan et al., 2015; Zainuddin et al., 2012).

1.4.2. Somaclonal variation

It has long been known that large variations sometimes occur in vegetatively propagated clonal plants, under identical environmental conditions. This has historically been noticed in sugarcane, potato and banana plants, and sometimes occurs beneficially, in cases like the navel orange (Bairu et al., 2011; Yang et al., 2010). This variation has been termed as somaclonal, and is often detrimental to goals of scientists, farmers and plant breeders, who expect uniformity within clonal populations.

Somaclonal variation occurs more often *in vitro*, where micropropagated plants are subcultured in media frequently, and in high numbers, as compared to field plants (Bairu et al., 2011; Larkin & Scowcroft, 1981). Some of the supposed causes of somaclonal variation, particular to plants in tissue culture, include wounding, exposure to sterilants (such as antibiotics), plant regeneration from incomplete tissue, artificial media compositions (with high sugar concentrations and plant growth regulators), lighting conditions and a high humidity within enclosed environments (Krishna et al., 2016; Sato et al., 2011; Us-Camas et al., 2014). Under these conditions, cultured plants sometimes undergo alterations to generate different cell types more conducive to the environment they are being kept in (Kitimu et al., 2015). Highly differentiated tissues such as roots, leaves, and stems generally produce more variations than explants with pre-existing meristems, such as axillary buds and shoot tips (Duncan, 1996). Tissue cultures maintained for long periods of time *in vitro,* result in greater somaclonal variations. Somaclonal variation within plants is genotype specific, and transposable elements already within the plants genome may result in additional mutations (Hirochika et al., 1996; Krishna et al., 2016).

Oxidative stress is one of the primary molecular mechanisms influencing somaclonal variation, and usually occurs as a response to abiotic stress in plants. Oxidative stress results in elevated levels of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl, peroxyl and alkoxyl radicals, which may alter the epigenetic methylation patterns of the plant DNA (Miguel & Marum, 2011; Wachsman, 1997). ROS also result in DNA and chromosomal mutations, such as base deletions, pyrimidine dimer formations, cross-links, strand breaks and base modifications, such as alkylation and oxidation (Gill & Tuteja, 2010). Both tissue culture initiation and subculturing expose plants to oxidative stress, with processes that modify plant cells, such as callus or protoplast formation, being especially stressful (Hirochika et al., 1996; Krishna et al., 2016).

The regeneration of plants from callus requires de-differentiation to form the callus, then re-differentiation to reform the organs of the plant. These conditions, cause plants to change their molecular compositions multiple times, and some of these processes may occur incompletely (Kitimu et al., 2015).

In cassava, inducing FEC and performing agrobacterium-mediated transformation with the regeneration of transgenic shoots takes between 20 and 30 weeks and has a high chance of resulting in somaclonal variation (Bull et al., 2009). Older FEC lines have a reduced ability to regenerate plants and often show somaclonal variation, leading to the recommendation that they be micropropagated for no longer than 6 months (Bull et al., 2009; Raemakers et al., 2001). Raemakers et al. (2001) make the case that somaclonal variation can alter transgenic plants to the point that they do not meet the agricultural requirements needed for sustainable production safety, leading to extensive selection procedures.

1.4.3. Post-transcriptional gene silencing

RNA interference (RNAi), which is mediated by small RNAs, is involved in many processes, such as the regulation of gene expression, suppression of invading viruses and protection of the genome (Mallory & Vaucheret, 2006; Pooggin, 2017; Yang & Li, 2018). The two main categories of small RNAs are micro RNAs (miRNAs), derived from long, single-stranded RNAs (ssRNA) and small interfering RNAs (siRNAs), that are derived from long, double-stranded RNAs (dsRNA) (Mallory & Vaucheret, 2006). Post transcriptional gene silencing (PTGS) is initiated when dsRNAs, produced externally, such as by viral pathogens, or internally, such as by cellular encoded RNA-dependent RNA polymerases, are processed to siRNAs by cellular Dicer and Dicer-like enzymes (Sherman et al., 2015; ***figure 1.7***). The siRNAs, which are usually between 21-24 base pairs in length, complex with other proteins into an RNA-Induced silencing complex (RISC) which unwinds and separates the two RNA strands (Sherman et al., 2015). This allows for the specific base pairing between the small RNA and the targeted mRNA, and subsequent degradation by exonucleases. This ultimately leads to a reduction in the expression of the targeted proteins, and in the case of viruses, some degree of resistance.

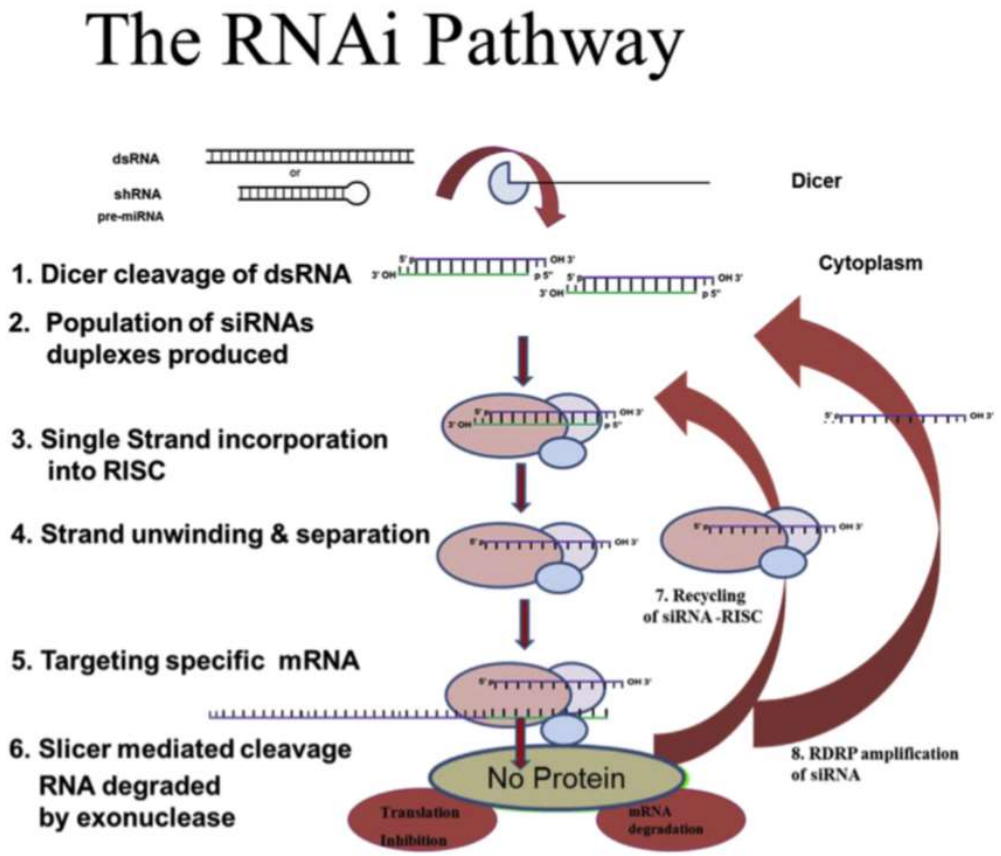

Figure 1.7 **The RNAi pathway**

(1) Long dsRNAs - derived internally from miRNA or externally from invading viruses, are cleaved by the Dicer endonuclease to small double stranded siRNAs. (2) The siRNAs duplexes generated complex with other proteins into a RISC. (3) RISC unwinds and separates the two strands. (4) The single strand siRNA-RISC complex targets a specific mRNA by specific base pairing between the siRNA and the targeted mRNA. (5) This leads to the elimination of the specific mRNA by enzyme cleavage or suppression of translation. (6) The corresponding proteins are prevented from being translated; the siRNA-RISC complex is recycled. (7) There is a process of amplification of the small pool of siRNAs by RNA-Dependent RNA polymerase (RdRp). (8) This results in the accumulation and intercellular spread of the RNAi molecules. (Sherman et al., 2015)

Engineering virus resistance by means of RNAi is preferable to viral protein expression mediated responses. The expression of full-length or truncated ACMV proteins in cassava can have deleterious effects on plant development due to interactions with plant proteins that function in the control of gene expression and defence (Zhang et al., 2005). Using transgenes that express long self-complementary hairpin RNA (hpRNA) to artificially induce RNA silencing in plants has been investigated as far back as 20 years ago by Waterhouse et al. (1998). The hpRNAs are highly effective at inducing RNAi in the plants

and have been widely employed since. The hpRNA constructs generally contain a sense and an antisense sequence from the gene that codes the target mRNA. The sense and antisense sequences are arranged as inverted repeats (IR), and are separated by a non-complementary spacer region, which stabilizes the hpRNA construct during cloning (Guo et al., 2016). If the spacer is a splicable intron, RNA silencing efficiency has been shown to increase (Smith et al., 2000).

In the case of geminiviruses, natural silencing is either through transcriptional gene silencing (TGS) via methylation of viral DNA, or by PTGS targeting of fold-back or aberrant mRNA, or dsRNA created by RNA-dependent RNA polymerase 6 extension of transcribed mRNA from overlapping reading frames (Vanitharani et al., 2005). Plant viruses, including geminiviruses, employ counter defence mechanisms, by encoding proteins that can supress RNA silencing. The DNA that codes these proteins is therefore an essential target of effective engineered RNA silencing. The replication associated genes (Rep) encoded by the AC1 ORF of the viruses are primary targets, as silencing them will inhibit viral replication. Geminivirus resistance has previously been reported in transgenic bean, tomato and cassava by targeting Rep with hpRNAs (Bonfim et al., 2007; Fuentes et al., 2006; Vanderschuren et al., 2009).

1.4.4. Social impact of genetically modified crops

As of 2018, there have been two decades of commercially cultivated genetically modified (GM) crops in South Africa. A combined 2.73 million hectares of land are dedicated to growing GM crops in South Africa (as of 2017), placing it ninth in GM crop production by area of land used (ISAAA, 2017). Despite the large adoption of GM crops, there are still some concerns that need to be addressed before GM crops can fully be embraced in the country.

A large number of consumers express concern over GM crops for human consumption, and the process of genetic modification in general. Many of these concerns are unfounded. A survey by Fernbach et al. (2019) found that as extremity of opposition to and concern about genetically modified foods increases in adults in the United States, France and Germany, objective knowledge about science and genetics decreases, but perceived understanding of genetically modified foods increases. GM crops are often considered as bad for the environment, by consumers. In many cases, transgenic crops reduce the amount of pesticides and herbicides applied by farmers, making them less hazardous to field workers and the

environment (Banta & Montenegro, 2008). This highlights the need to educate consumers on the mechanisms of genetic modification, and the benefits of GM foods.

The driving force in producing GM crops is economics, leaving it largely in the hands of multinational companies, who prioritise the production of the most profitable plants (Valentine, 2003). It also forces GM crop researchers to address matters of intellectual property in addition to regulatory processes involved in environmental and food safety (Taylor et al., 2004). Most plant biotechnology is focused on traits that benefit farmers in industrialized regions of the world, but this trend is changing in favour of modified crops that contribute to enhanced ecological and human health (Banta & Montenegro, 2008), such as with "Golden Rice," rice (*Oryza sativa*) enriched for the vitamin A precursor, B-carotene (Beyer et al., 2002). In GM cassava research, vitamin fortification (Adenle et al., 2012; Li et al., 2015; Mangel et al., 2017; Sayre et al., 2011; Vanderschuren et al., 2013); and starch structure and enhancement studies (Bull et al., 2018; Ihemere et al., 2006) have also been reported.

The elite, high-yield cassava cultivars that farmers prefer, grow straight, are late-branching, flower poorly and are adapted to the environmental conditions they are grown in (Fermont et al., 2009; Lebot, 2009). These cultivars are often recalcitrant to current methods of transformation, and genotype-independent transformation procedures are highly sought after (Zainuddin et al., 2012). Chetty et al. (2012) experienced success in transforming cv. T200 cassava, a high yield farmer preferred landrace. There is also the need to modify these plants without inadvertently decreasing the quality of the plant and its tuber yield, by effects such as somaclonal variation. Methods of utilizing RNAi in cassava for virus resistance have met roadblocks, such as viral suppressors of silencing (Burgyán & Havelda, 2011) and populations of geminiviruses diversifying in targeted areas to avoid silencing (Mehta et al., 2019a). It has also been found that CRISPR modified virus-resistant cassava plants lose effectiveness over time in the field (Mehta et al., 2019b).

1.5. Salicylic acid effect on tuber growth

Koda et al. (1992) tested a variety of plant growth regulators, to determine which had the greatest effect on tuber formation in potato plants (*Solanum tuberosum*). They found that when applied as a foliar spray, salicylic acid (SA) caused the earliest initiation of tuberization, at the lowest concentrations, followed by acetylsalicylic acid (ASA). This effect was presumed to be caused by derivatives of benzoic acid with a free carboxyl group and

substituent at the C-2 position of the ring, which may promote the biosynthesis of tuberonic or jasmonic acid, that have known tuber inducing activity. Many more studies subsequent have shown that SA and ASA improve the production of tubers, at the expense of shoot production, in potato plants and a variety of tuber producing plants such as carrot, beetroot and radish plants (Larqué-Saavedra & Martin-Mex, 2007; Lopez-Delgado & Scott, 1997).

It is unclear if the same tuber inducing effect occurs in cassava when treated with SA. Duque and Setter (2013) noted that ABA accumulated in cassava at the onset of water stress. ABA has been found to promote changes that help plants cope in water stressed environments, by means of reducing shoot growth, leaf area expansion and transpiration, as well as stimulating root growth (Alves, 2002). SA often plays a role in early plant responses to disease, though there is evidence that this might not be the case in cassava (Allie et al., 2014).

1.6. Nonlinear modelling for plant growth

A model is a mathematical interpretation of a system or process, which can help to describe or predict that system. Depending on the type of model, it may, or may not have a predictive or explanatory purpose (Van Sanford, 2005). In situations of modelling biological growth specifically, it has been determined that complex, nonlinear functions are necessary (Fekedulegn et al., 1999). These theoretical models have an underlying hypothesis associated with cause or function described by the response variable, often suited to a specific biological phenomenon. This is advantageous over empirical models, such as polynomial and mixed linear models, which fit the trend of the data, and have a low predictive power beyond the range of data.

Growth models are often represented as a distinct formula or a set of formulae, often named after the researcher or researchers that originally described them, or their original usage. ***Table 1.1*** below shows common growth model formulae used for modelling crop growth; with their sources, where ω is the dependent growth variable, t is the independent variable (usually time), α, β, k, and m are parameters to be estimated, exp is the base of natural logarithms, and ε is the additive error term (Fekedulegn et al., 1999). The parameters determine the shape of the curve when the model formula is plotted as a function of the dependant growth variable. The parameters often have a biological meaning or significance to the growth of the organisms or subject modelled. There are multiple forms of these equations for each model, though their use is based on the biological system they are

monitoring. Because the parameters estimated are in nonlinear equations, they are more difficult to specify and estimate than linear models and the solutions are determined iteratively (Panwar, 2012).

Table 1.1 Common nonlinear models used with crop growth

Model	Formula	Source
Beta	$\omega(t) = c+(\alpha-c)+(1+(\beta-t)/(\beta-k))((t-m)/(\beta-m))^{(\beta-m)/(\beta-k)}+\varepsilon$	(Yin et al., 2003)
Von Bertalanffy	$\omega(t) = \alpha(1-e^{-k(t-\beta)})^{m}+\varepsilon$	(Archontoulis & Miguez, 2015)
Gompertz	$\omega(t) = \alpha \exp(-\exp(-k(t-\beta)) + \varepsilon$	(Archontoulis & Miguez, 2015)
Monomolecular	$\omega(t) = \alpha(1-\beta\exp(-kt)) + \varepsilon$	(Draper & Smith, 1981)
Logistic	$\omega(t) = \alpha/(1+\exp(-k(t-\beta)))+\varepsilon$	(Archontoulis & Miguez, 2015)

1.7. Rationale for study

Cassava does not currently play a large commercial role in South Africa but has the potential to be highly valuable to the country. The crop is grown alongside maize by small-scale and subsistence farmers in the Limpopo, Kwazulu-Natal, and Mpumalanga provinces. Cassava has an inherent tolerance to stressful environments, such as poor soil and droughts; that other food crops, such as wheat, rice and maize, fail to have (El-Sharkawy, 2003). The massive leaf production of cassava means that cassava requires little fertilization over time, as the falling leaves recycle soil nutrients. Additionally, cassava tubers can stay in the ground for up to three years, and cassava plants respond well to high carbon dioxide concentrations (Legg et al., 2014b). For this reason, it is known as a food security crop, and would be a beneficial additional food security source in South Africa, which has been recently facing drought conditions.

In addition to being a good source of food, cassava starch has many potential industrial uses, such as in animal feed, biofuel, paper, textile, and food processing applications (Howeler et al., 2013). Using the starch for biofuel can reduce the current reliance on fossil fuels as industrial energy sources (Adelekan, 2010, 2012). If successfully implemented, cultivating

cassava for industrial use can produce large economic gains in South Africa and other developing countries. To effectively develop cassava for small and large scale farmers for food and agro-processing, first the threat of infectious diseases, like CMD, needs to be addressed in order to reduce yield loss (Rey et al., 2012). CMD is caused by several geminiviruses, and in South Africa SACMV, ACMV and EACMV have been detected (Berry et al., 2004). In order to reduce geminivirus-induced yield loss, a genetic engineering (GM) approach was undertaken, and several transgenic RNAi lines were generated in the Plant Biotechnology Laboratory at the University of the Witwatersrand. Three of these transgenic lines will be evaluated in this study.

1.8. Aim of study

Three transgenic experimental cassava lines that were found to be tolerant to ACMV (Mvududu, 2017), were investigated for storage root/tuber production in laboratory conditions. These are the CMM6-2, CMM6-6, and AMM2-52 lines, which are based on cassava cv.60444 transformed with ACMV [Nigeria: Ogorocco;1990] derived RNAi hairpin constructs. The transgenic lines from which these tolerant cassava lines were selected, were engineered by Moralo (2015). The constructs were inserted into the model cassava cultivar cv.60444 by agrobacterium-mediated transformation. The CMM6 lines are transformed with a non-mismatched inverted sense-antisense DNA repeat construct which transcribes a hairpin (hp)RNA (MM6hp) derived from the overlapping ACMV AC1/AC4 (2437-2572 nt) and AC2/AC3 (1297-1479 nt) viral open reading frames. The MM6hp construct was made stable by positioning an intron between the IR fragments. AMM2 lines contain a mismatched hpRNA construct (MM2hp) of the same viral sequences. The replication initiation protein (AC1), transcription activator protein (AC2), replication enhancer protein (AC3) and viral silencing suppressor protein (AC4) coding mRNAs of the DNA-A component are all intended targets for silencing by siRNAs generated by dicer-processing of the hairpin/dsRNA. The mismatch was introduced into the MM2hp by sodium bisulphite deamination-induced mutations in the hairpin sense-arm (Taylor et al., 2012b). This was done to reduce the complementarity between the sense and anti-sense arm, which enhances IR stability, and prevents undesirable cruciform junction formation in the hpRNA formed (Moralo, 2015; Taylor et al., 2012b).

The aim of this study is to perform six month pot trials of two MM6 and one MM2 ACMV-transgenic lines to evaluate tuber yield, in comparison to wild-type untransformed cassava

plants and a known transgenic ACMV resistant line (dsAC1, provided by H. Vanderschuren). Furthermore, different experimental conditions were tested to optimize tuber formation, since information on tuber induction in pot-trials for cassava was not available from the literature.

1.8.1. Objectives

- Perform three cassava trials in controlled plant growth facility
    - Agroinoculation of plantlets with ACMV and mock inoculation (first trial)
    - Planting in large and small pots (second trial)
    - Foliar spray with salicylic acid compared to no salicylic acid (third trial)
- Screening or evaluation at end of trial (six months):
    - Tuber dry and fresh weight
    - Starch content (total starch quantification) of the tubers harvested from the trials
    - Starch analyses (amylose/amylopectin ratios, polarized light microscopy)
    - Model and evaluate plant characteristics: height and number of leaves

Chapter 2: Methods and materials

2.1. Cassava lines

CMM6-2, CMM6-6 and AMM2-52 lines described in Chapter 1 were evaluated for tuber properties. For comparison, two untransformed controls were used, namely the CMD-tolerant TME3 landrace and susceptible cv.60444 cultivar (model cultivar used for transformation). A known resistant ACMV-transgenic cassava line (dsAC1) was used as a positive control. The dsAC1 cassava line was engineered by Vanderschuren et al. (2009), by transforming cv.60444 cassava to express an AC1-hairpin dsRNA homologous to ACMV [Nigeria: Ogorocco;1990] AC1/Replication associated protein (Rep) sequence from the 1690 to 1844 nucleotide position.

2.2. Experimental design

Three plant trials were conducted for these three transgenic lines. The cassava plants were grown in plant growth facilities where conditions such as light intensity and temperature were controlled. For the first trial, 12 of each transgenic line and control were potted in small 9 cm square pots, with 6 of the 12 plants being inoculated with ACMV. The second trial had half of the 12 plants of each line or cultivar potted in larger 15 cm round pots, and the other half in small 9 cm pots. In the third trial, plants were grown in 9 cm pots, with half of the plants sprayed with a salicylic acid (SA) solution, and the other half sprayed with water. Additional plants were cultivated in each trial (approximately 20 of each line/cultivar), in an attempt to account for plant mortality. Conditions were kept as controlled as possible within each trial, by means such as using the same amount and type of potting soil, fertiliser, and water in all pots. The plants of every cassava line/cultivar were also grown at the same time and with the same tissue culture conditions. Light intensity, temperature and soil moisture were measured in the plant growth facility. A transparent Perspex box was used to grow a few plants in the first trial, in an attempt to view the progress of tuber growth.

The height and number of leaves of the plants were recorded monthly throughout the experiment, for use in growth modelling. Six months after potting, the tubers were harvested in each trial and analysed. Wet and dry weight ratios, total starch content and amylose/amylopectin ratios were measured on the largest tubers (at least 2 tubers from each treatment of each line/cultivar, depending on availability) from the control and test subjects of each trial.

2.3. Bulking up and acclimatisation of cassava

Cassava stocks were grown in glass jars, in Murashige and Skoog medium with a 20g/L concentration of sucrose (MS20) and 7.8g/L concentration of agar. The stocks for each trial were started on the same day by nodal propagation to limit variation. Nodal cuttings were then micropropagated onto MS20 plates with a 6.8% plant agar concentration and stored at 28℃ under 16 h light/8 h dark photoperiod. Approximately 25-30 of each cassava line were propagated, to account for plants that would perish before and during the trial.

Once the plantlets had developed adequate roots, usually around three to four weeks they were transferred to small Jiffy 7 bags (Jiffy Products Ltd.). The Jiffy bags were placed in deep plastic trays and covered with plastic wrap then stored at 28℃, 16 h light/8 h dark photoperiod and 60% humidity. After one week, holes were poked through the plastic wrap to help acclimatise the plants.

2.4. Growth conditions of plants

After the plants had been acclimatised in porous Jiffy bags, they were transferred to 9×9×9.5 cm square pots. In trial 1, the Jiffy bags were slit on three sides to allow for root and tuber growth. The potting media was composed of a 2:3:3 mix of river sand, all-purpose potting media, and vermiculite. Additional transparent Perspex cases were included in the first trial to grow some of the plants in an attempt to track the growth of the tubers.

These conditions proved to be sub-optimal for tuber growth, so were altered for the second and third trial. The Jiffy bags were completely removed from the soil pellet, before the plants were potted in the 9×9 cm pots, to allow for unrestricted tuber growth. The potting media was altered to a composition of 1:3 perlite and seedling mix. Vita Veg Vegetable & Herb Fertilizer, with an NPK grade of 6:3:4 (Nitrogen, Phosphorous, Potassium ratio), was used to fertilise the plants monthly. This fertilizer has a low phosphorous content, so should promote tuber growth over leaf and flower growth. A fixed amount of the solid fertiliser, the equivalent of 6 pellets was added to each pot.

The plants were grown in a controlled basement plant facility, as shown in ***figure 2.1***. The temperature was regulated to 28℃, and humidity at 60%. The plants in square pots were kept on shallow plastic trays, on metal shelves equidistant (410cm) to a light source made up of a cool (4000k) white (Osram L58W/640) and purple (Sylvania Gro-lux F58W/Gro T8) plant-growth fluorescent lights, and a 16 h light/8 h dark photoperiod. The light intensity was

measured at the start of the trial, at the leaf canopy of each of the plants, to ensure consistent lighting, and between the trays of the plants. At zero days after potting (DAP), light intensity measured an average of 21.99 μmol m^{-2} s^{-1} (s.d. 4.34) at the canopy of the plants. There was a small amount of variation, with a slightly higher light intensity for plants in the centre of each tray. Light intensity was also measured above each of the plants sporadically during the trial, to ensure that the lighting was consistent throughout the trial.

Plants were watered twice a week, with tap water. Each plant, of each line/cultivar was watered with the same amount of water, from the surface of the soil, throughout the trial measured with a 1L graduated cylinder. A rudimentary soil moisture sensor (YL-69) connected in analogue to an Arduino Uno Rev3 was used to measure the moisture in the soil every month, ensuring that the amount of water provided for each plant did not result in any of the soil in the pots drying out. When a reading of -50% or lower before watering was found on the moisture sensor, for any plant, the volume of water was increased for all plants in the trial. The intention was to slightly under water the plants, as water stress is a factor proposed to increase tuber production in small pots. Soil moisture readings in a pot between 30-40% the day after watering, usually indicated ideal soil moisture. This usually resulted in a starting watering volume of 50 ml per plant twice a week in the beginning of each trail, to 150 ml at the end of the trial. Since all plants were watered with the same amount of water each, in each trial, plants that consumed more water (ostensibly larger plants) would be under a greater water stress than plants that consumed less water. The recorded soil moisture levels were tested against tuber and plant characteristics, to determine if there were any correlations present.

Figure 2.1 **Cassava from trial 2 grown in basement facility**

2.5. Trial sub-treatments

2.5.1. Agroinoculation with ACMV in trial 1

Three weeks after transfer to the Jiffy bags, half of the cassava plants from each line were agroinoculated with ACMV[NG:Ogo:90] DNA-A and DNA-B infectious clones (Moralo, 2015; Mvududu, 2017). The Agrodimers A and B cloned in a pCAMBIA 1300 vector, were transformed into *Agrobacterium tumefaciens* LBA4404 then grown separately overnight in Yeast extract peptone broth inoculated with 100 µg/ml streptomycin, 50 µg/ml kanamycin and 50µg/ml rifampicin antibiotics until an OD600 of between 1.8 and 2.0 was reached. Acetosyringone was added to the culture, to a final concentration of 200 µM. Each plantlet was injected at the nodes with 120µl of *Agrobacterium* culture containing equal volumes of ACMV [NG:Ogo:90] DNA-A and B dimers. The remaining plants were mock-inoculated with *Agrobacterium tumefaciens* LBA4404 containing an empty pCAMBIA 1305.1 vector, in the same process as the infected plants. Infection of the ACMV agroinoculated plants was verified by examining the leaves of the plants for symptoms of infection at 32 days post infection. The symptoms examined were signs of leaf mosaic with or without leaf deformation, on the second and third upper-most apical mature leaves, in comparison to the control mock inoculated plants.

2.5.2. Potting in large and small pots in trial 2

During the first trial there was a high mortality of infected and mock infected plants, so alternative sub-treatments were introduced in trials 2 and 3, with the aim of improving tuber production. There was no infection with ACMV in the subsequent two trials. In addition to the 9cm square pots, some of the plants were potted to larger 15 cm diameter, 12.5 cm high, round pots (usually three pots per line or cultivar). For this trial, plants in the 9 cm square pots are labelled as small pots, in comparison to the larger 15 cm diameter pots, labelled as big pots. These pots were watered with larger volumes of water. The increased water volume was determined by altering the amount of water added until the soil moisture was approximately the same as the small plant, after watering, at the start of the trial. This resulted in a final watering value of 200 ml of water per large pot at the end of the trial. Due to the larger size of the pots, these plants were 2.3 cm closer to the light source than the smaller pots.

2.5.3. Spraying with a salicylic acid solution in trial 3

During the third trial, plants were treated as in trial 2, but sprayed with a salicylic acid solution. Half of the plants of each line/cultivar were subjected to a foliar spray of the salicylic acid solution, the remaining half with a tap water solution, every two weeks. The salicylic acid solution was freshly prepared before each spray, by diluting 1M salicylic acid dissolved in 99.9% ethanol, to a 5×10^{-5}M with tap water. This was increased to a 10^{-4}M solution, with an addition of 0.01% Tween 20 (polysorbate 20) halfway through the trial, to aid in leaf absorption. Each tray of 12 plants was and sprayed with 12 ml of the salicylic acid solution or tap water solution, with a spray bottle.

2.6. Plant measurements

The height and number of leaves of each plant were measured monthly and recorded. The main-stem height, measured to the nearest millimetre, was taken from the base of each plant, to its apex. If the plant had not grown vertically in a straight line, it was carefully lifted, without stretching the plant. Originally a 30 cm flexible ruler was used, replaced by a 5 m tape measure, as the plants grew taller. Some plants had grown multiple branches or stands as they grew taller. These were recorded, but the tallest stand or branch was used to determine the plant height. The cassava plants grew more brittle as they aged, sometimes resulting in broken main stems, which were also recorded and taken into account. The number of leaves

on each plant was counted manually, excluding leaves which had not fully expanded and senescent leaves.

2.7. Tuber analysis

2.7.1. Dry/wet weight

After the final plant measurement, six months after potting, the tubers were harvested. The tubers were pulled out of the potting media and removed from the base of the plant. Adventitious roots attached to the tubers were also removed. The tubers were washed with tap water, then a 70% ethanol solution, removing the periderm, but leaving the cortex intact. Each tuber was then accurately weighed on a laboratory balance in grams to five significant figures. The plant material above the potting media from each plant was weighed, by placing the plants on a measuring cylinder above the balance. The tuber mass and plant mass above the ground was used to calculate the harvest index (ratio of harvested tubers to above ground plant mass).

The tubers were labelled and stored at -80 ℃ in plastic bags. To measure the dry to wet weight ratio, and prepare the tubers for further tests, half of each tuber to be measured was grated finely, weighed, then dried at 80 ℃ for 48 hours. The dry mass of each was then measured and used to calculate the dry to wet ratios. Where necessary, the dry material was further crushed, or ground to a fine powder, or flakes, for use in further starch tests.

2.7.2. Amylose/Amylopectin content

The Megazyme Amylose/Amylopectin kit (K-AMYL 06/18) was used to determine the ratio of amylose to amylopectin in the largest tubers from each line/cultivar and treatment. The amount of glucose oxidase plus peroxidase (GOPOD) reagent used in the protocol was altered from 4ml to 3ml, for easier use with total starch measurements. Each test included 3 technical replicates, with additional repeat tests from a second sample of every fifth tuber.

2.7.3. Total starch assay

Total starch contents of the tubers were estimated using measurements from the Amylose/Amylopectin Assay, with the Megazyme Total Starch calculator (K-TSTA_CALC). Each test included 3 technical replicates, with additional repeat with additional repeat tests from a second sample of every fifth tuber.

2.7.4. Starch granule observation

Tuber material was scraped off the centre of tubers of each line/cultivar from trial 1 and 2, with a sterile scalpel, and placed on a water droplet upon a glass slide. A cover slip was placed atop the water droplet, surrounded by glycerol to prevent evaporation. This was then viewed with a Zeiss Axio Imager M2 microscope at 400× magnification with differential interference contrast (DIC) microscopy imaging. The images taken, were analysed using a scale bar, to confirm that the characteristic shape of the cassava starch granule, and observe the birefringence present. The average size of three large, small and middle-sized starch granules was also measured, using ImageJ (Rueden et al., 2017).

2.8. Data analysis

Data was recorded and organised in Microsoft Excel and exported to the R statistical program, version 3.6.1 (R Core Team., 2014) for analysis and figures were produced using ggplot2 (Wickham et al., 2016). To correct for height anomalies in modelling, wherever a broken main stem was recorded, that height entry was removed, and future measurements would take into account the rate of growth above the mean after that point.

2.8.1. Least square means, analysis of variance and Pearson correlation

Data was first tested for normality with boxplots and Shapiro-Wilk tests. Least square means, otherwise known as estimated marginal means, were calculated for plant height and number of leaves. These were grouped by treatments and plant lines/cultivars, at each time point (measured monthly) for each trial. This was calculated using the emmeans function (Lenth, 2019) in R. Two-way analysis of variance (ANOVA), with type 3 sum of squares, was performed on the same groups, followed by pairwise t-tests between each group with Bonferroni correction. These results were tabulated with standard deviations. Pearson correlation tests were performed to test the correlations between tuber characteristics and plant growth data at the end of the trial. The characteristics involved were: number of tubers, tuber weight, tuber amylose content, tuber starch content, tuber moisture content, harvest index, light intensity at the start of the trial, plant height at the end of the trial, plant leaf number at the end of the trial, number of branches/stands at the end of the trial and plant mass at the end of the trial. Plant growth characteristics and conditions were also tested for correlations at each point, namely: leaf number, height, number of branches/stands and soil moisture.

2.8.2. Nonlinear models

The growth rates of the plants were characterised using nonlinear growth models. The growth characteristics measured were plant height and leaf number, for each treatment of each line/cultivar in trial 2 and 3. A nonlinear model is used for these characteristics, as it has been determined that complex, nonlinear functions are necessary to accurately model growth in biological studies (Fekedulegn et al., 1999). The model fit was performed using nls2 (Grothendieck, 2013) for least squares estimation with brute force, in R. The resulting parameters were tabulated and plotted with scatter plots of their associated data. For models with a known timepoint for maximum growth rate, the maximum growth rate was determined from the first derivative of the model and recorded. Goodness of fit statistics were obtained from the residuals of each model, to analyse the fit of the models to the data. The goodness of fit statistics presented are the root mean squared error (RMSE), Akaike's information criteria (AIC) and Bayesian information criteria (BIC). The BIC was used the most in comparing models, as it strongly penalizes additional unnecessary parameters in a model.

The models used are presented in ***table 1.1***. The Beta, Gompertz, logistic and Von Bertalanffy model were selected for plant height, as they are sigmoidal in shape, which is associated with plant growth. For leaf number, the Beta, Monomolecular and Von Bertalanffy model were chosen, as the expected trend of this data is not known. The Monomolecular model has a shape that decreases in slope until a y-asymptote is reached. The Beta model is sigmoidal, up until it reaches the y-asymptote, where it has the capacity to curve downwards. To allow for more specific parameter estimates, the β parameter of the Beta model was set at -60, the approximate time when the plants were propagated.

2.9. Flow diagram of the experimental approach

The following diagram summarizes the methods discussed in this chapter.

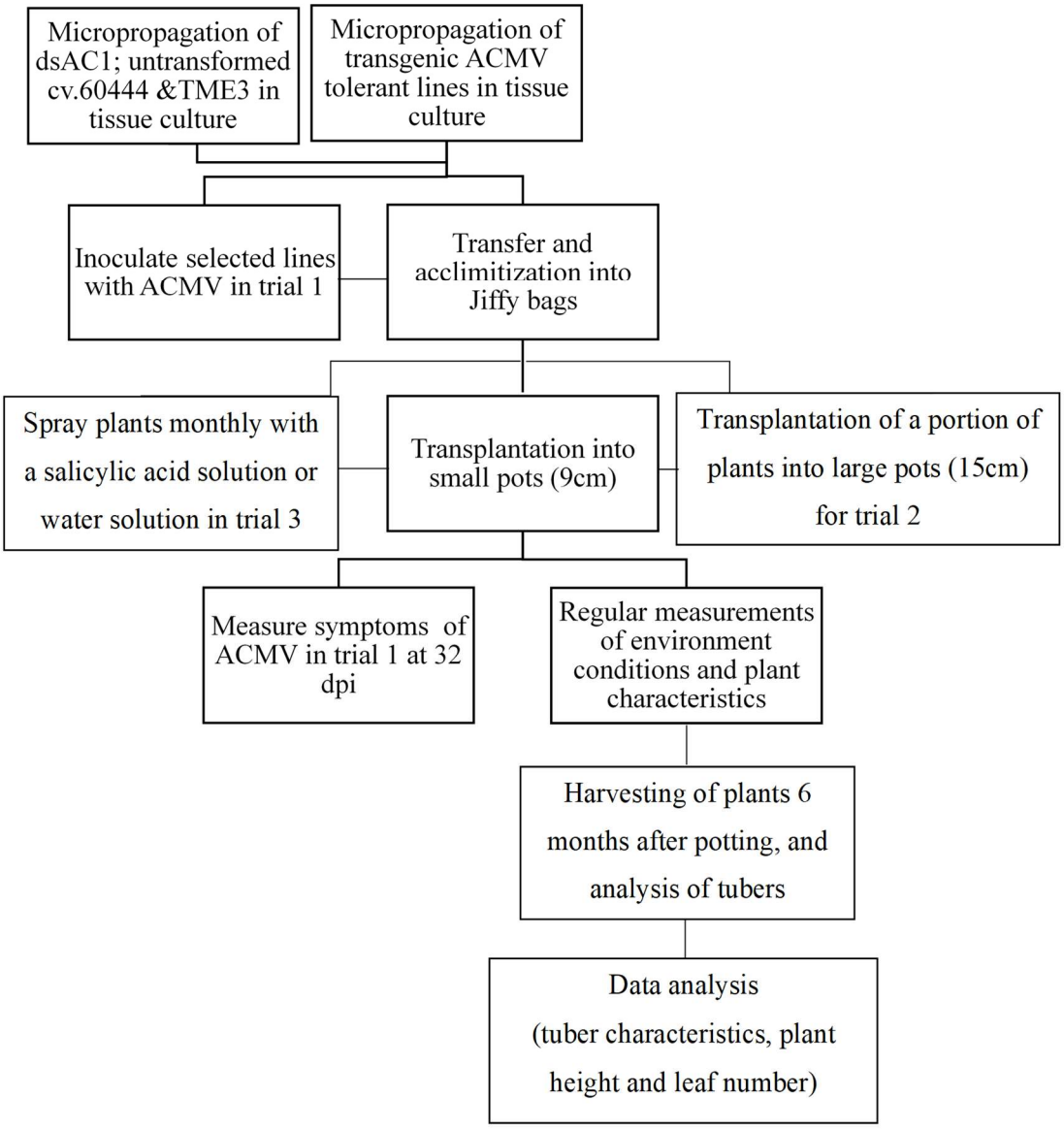

Figure 2.2 **Flow diagram of the experimental approach**

Chapter 3: Results and discussion

Research on cassava is often focused on tuber production, as it is the primary commodity of the plant. When transgenic cassava lines are produced, it is necessary to perform tests to evaluate the potential effects on the tubers. There are many potential sources of variation in the process of developing transgenic plants that can result in altered tuber production or composition. The process of FEC production and *Agrobacterium* mediated infection could result in somaclonal variation. There may also be unintended effects from the integration of the transgene into the plant's genome and the expression of the transgene. These factors may impact the expression of genes related to the tubers (Kitimu et al., 2015; Latham et al., 2006). Consequently, this study aimed to compare transgenic lines with non-transgenic cassava plants and the dsAC1-ACMV tolerant line, in order to evaluate if transformation had any effect on tuber production.

For preliminary studies, before field trials are performed, cassava is grown in highly controlled laboratory environments with sub-optimal conditions. These conditions make production of tubers difficult with cassava plants, as stimulation of tubers often requires specific field conditions, such as soil, lighting, temperature, and water availability. Consequently, the transgenic ACMV tolerant cassava plants in this study were grown under non-field controlled growth room conditions, with sub-treatments that could potentially allow or enhance tuber production and their growth, in order to be able to optimize tuber production in transgenic vs non-transgenic plants in six month trials.

3.1. Starch granule analysis

The starch granules analysed were taken from scrapings of the centre of the tubers, without standardised dilutions, to focus on the morphology and size of the granules. There was very little processing involved, so the size, shape and composition of the resulting granules are unlikely to have been altered. Results from this study showed that granules from all the cultivars and transgenic lines measured from trial 2 were round shaped, with some being slightly truncated on one side (as exhibited with results from cv.60444 cassava in ***figure 3.1)***. There are a few other possible shapes for the starch granules in cassava, but the shape of these granules is consistent with what was found in other studies with tubers from young plants (Vasconcelos et al., 2017).

The averages of the diameters of three large, medium, and small granules were taken for all samples of trial 2 (***table 3.1***). These were all within the same size ranges, as each other, for each size group with large granules ranging between 13 and 19 µm, the medium sized granules ranging between 8 and 14 µm, and small between 3 and 6 µm. There were no granules observed with irregular size or shape, in any of the lines or cultivars. The size of the medium and large starch granules was consistent with what has been found in other studies with young tubers (10.0 to 17.0 µm diameter), though the small granules were smaller than usual (Vasconcelos et al., 2017). The smaller granules were most likely present due to the young age of tubers, as the size distribution varies with the stage of development of the plant, and the newly formed granules might not have swelled with starch yet (Leonel, 2007). The size and shape of the starch granules is very important in determining some of the commercial uses of the starch, such as the production of paper biodegradable plastic films (Leonel, 2007).

The starch granules of all the transgenic lines, non-transformed cultivars and dsAC1 transformed control all display the characteristic birefringent cross under polarised light (as observed in ***figure 3.1*** for cv.60444 cassava granules). The birefringence is caused when the polarised light is reflected twice, due to crystalline structures in the granule. Crystalline structures form due to the radial arrangement of amylopectin molecules, and the 90° angles their chains form with the starch granule centre or hilum (Alcázar-Alay & Meireles, 2015). Weaker birefringence can occur in the starch granule with greater ratios of amylose, which has an amorphous structure (Singh et al., 2003). The amylose within the starch granule can vary between plants of the same species grown in different conditions. There were no noticeable differences in the shape or intensity of the birefringence crosses found in the starch granules observed in this study. The uniformity of the features of the starch granules tested indicated no conspicuous differences of the starch of the transgenic ACMV tolerant lines with those of the control cultivars and dsAC1 line.

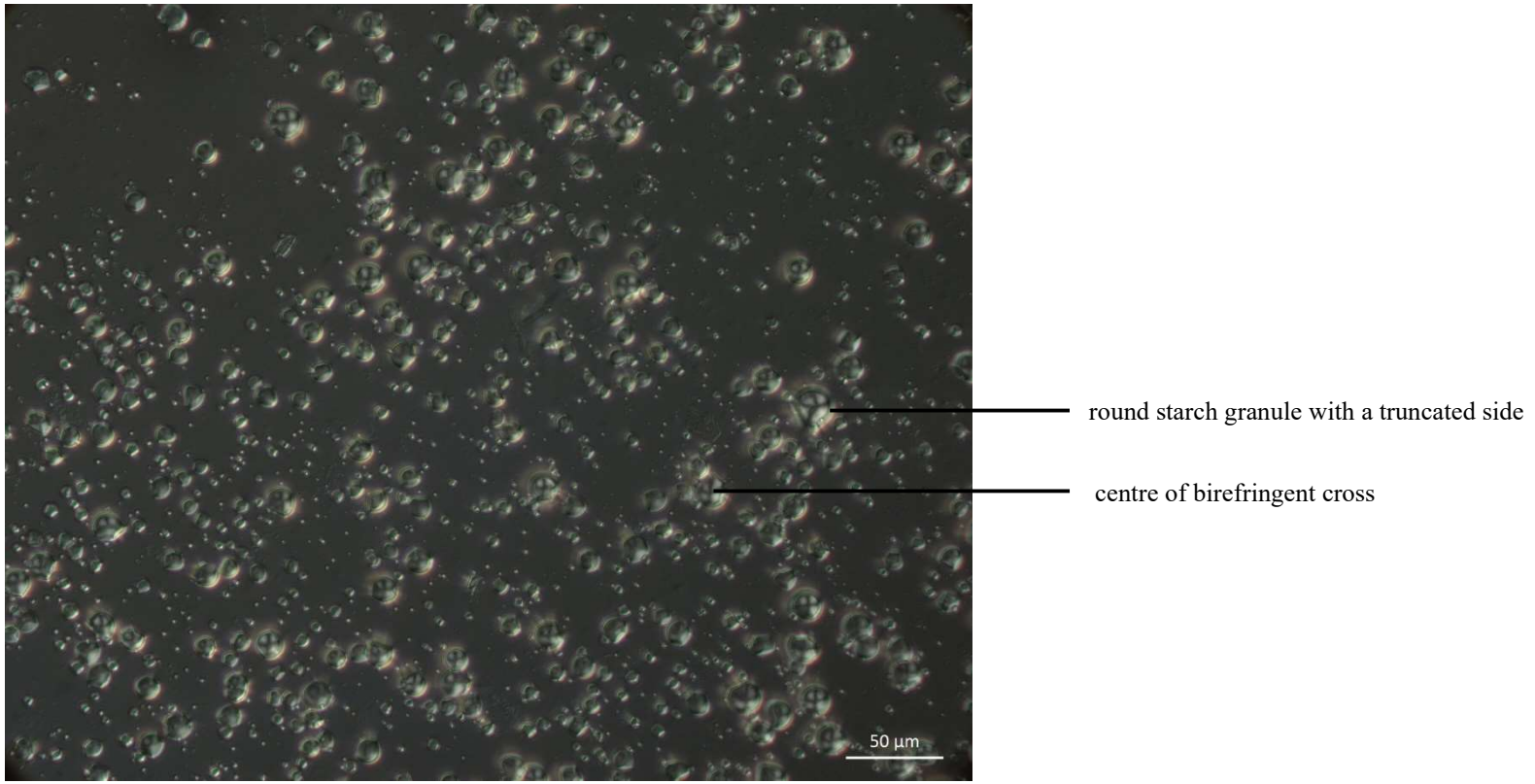

Figure 3.1 **Image of starch granules from cv.60444 cassava tuber, at 400x magnification, with DIC**

Table 3.1 Starch granule average diameters (µm), from tubers of trial 2

Line/Cultivar	Large granules	Medium granules	Small granules
AMM2-52	13.1(1.9)	8.6(0.7)	3.2(0.7)
CMM6-2	17.5(0.4)	13.9(1.7)	3.9(1.3)
CMM6-6	12.1(2.2)	10.6(0.4)	4.8(0.5)
cv.60444	18.2(2.0)	10.5(0.8)	4.9(1.0)
dsAC1	16.5(1.9)	10.9(0.1)	6.0(1.5)
TME3	15.0(1.3)	9.9(0.7)	3.9(0.8)

Mean granule size (µm) taken from 3 selected granules, (standard deviation)

3.2. Tuber production and characteristics

3.2.1. Tuber production

The process of agroinoculation with ACMV and mock inoculation with agrobacterium was highly stressful to the plants and resulted in a high mortality of plants early in the first trial. The subsequent two trials focussed on conditions that would promote tuber production and improve the number of plants that survive in the trial. ***Table 3.2***, column 3 displays the ratio of plants that produced tubers to those that did not. In trial 1, cv.60444 ACMV inoculated and CMM6-6 plants did not produce any tubers. For trials 2 and 3, CMM6-6 in small pots; water sprayed AMM2-52, TME3 and salicylic acid (SA) sprayed CMM6-2, CMM6-6 and cv.60444 plants did not produce tubers. For trial 3, of the lines/cultivars that did not produce tubers, all had a high number plants surviving to the end of the trial (as shown in the square brackets of column 3, ***table 3.2***) ranging between 7-10 plants for each group, excluding water sprayed TME (with 4 plants). This indicates that ideal conditions are required to produce tubers, and that between trials, slight changes in the environment of the study may affect tuber production. The TME3 landrace reliably produced tubers in trial 2, with all 4 plants (1×[4]) from large pots producing 10 tubers (***figure 3.3*** number of tubers weighed, where all fresh tubers are weighed in the trials), and nine plants (0.819×[11]) producing 20 tubers in (***figure 3.3***). This trend does not persist in trial 3, where one (0.111×[9]) of the SA sprayed plants and none of the four water sprayed plants produced tubers. In the case that the foliar sprays of trial 3 do not reduce tuber production for the TME3 landrace, it implies that a line or cultivar that produces a large number of tubers in a trial, might not do so in another, with similar environments.

It was expected that tuber production would be slow, since after three months of growth, signs of micro-tubers were shown to be present in field grown cassava (Alves, 2002). However, previous trials (Walsh, 2019) using 15 cm pots, in indoor growth facilities, were successful in producing tubers. There are many conditions inherent to indoor growth facilities that may have led to the low tuber production after six months, such as the artificial lighting or fixed temperature. It may be that the conditions of the growth facilities may have been optimal for cassava plant growth, providing no impetus for the plants to produce tubers. In some cases, in controlled environments, it has been found that the proportion of dry matter in the roots increased in low-temperature plants at a greater rate than plants kept at high temperatures (Mahon et al., 1976). In over-fertilised environments, cassava sometimes

allocates more resources to stem growth, to the detriment of tuber production (Fermont et al., 2009; Pellet & El-Sharkawy, 1993).

A more likely reason for the low tuber production was postulated to be a lack of space in the pots for roots to grow. In the Perspex which were used for observation of the roots/tubers, roots grew to the bottom (>30 cm) during the six months of growth (***figure 1.2B***). In some of the plants from all three trials, in large and small pots, a large amount of fine root growth was found when harvesting the tubers, sometimes covering the interior surface of the pot (***figure 1.2A***). This large growth of roots in a small space causes soil compaction, which can result in reduced cassava tuber yields (Maduakor, 1993). It may also lead to hypoxia of the plant roots, when they restrict other roots and prevent drainage of water. There was a slight water stress imposed when watering the plants, to reduce some of the effects of the constrained space, though this seems to have been ineffective. Taller pots may alleviate this effect, by allowing the roots of the cassava plant to grow deep, allowing more space for the tubers to grow, in a dryer area of the soil. However, this is often not feasible in indoor growth facilities with fixed lighting. In contrast, two other studies of cassava tubers in pots, in controlled plant facilities; smaller pots (9 cm) compared to 15 cm or large (20 cm) were shown to encourage plants to make tubers (personal communication H. Walsh and S. Bull) compared to large (20 cm pots). Optimising light conditions may also enhance tuber production. Lowe et al. (1976) found that a short daylength of less than 14 h (with 30 ℃ day 25 ℃ night temperatures) promoted the initiation and early growth of root tubers in the cultivar tested. Greater light intensity during early plant growth (107.82-134.78 μmol m^{-2} s^{-1}) and green house conditions later in growth may also prove successful in promoting tuber growth (Mvududu, 2017). Use of other fertilisers, like mineral fertilisers such as the Wuxal fertilizer, with trace elements and an NPK grade of 8:8:6, is another treatment that could improve the chances of tuber production (Bull et al., 2018).

The average fresh weights of the tubers ranged from 0.280 g to 4.344 g (***table 3.2***, column 4). The largest tubers found, just below 13 g in weight, were from dsAC1 and TME3 plants in big pots, of trial 2 (***figure 3.3***). All of the tubers harvested after 6 months potted were small, with none exceeding a height of 10 cm (***figure 3.2***). The harvest index (HI) values represent the ratio of tuber mass to plant mass above the soil. In the field, with high yield landraces, the HI for mature cassava plants is generally above 0.5, and below 0.7 (Sagrilo et al., 2008). The HI values were generally below 0.5, with the exception of some of the plants in trial 2, which had HI values exceeding 0.7, with TME3 plants in small pots having an HI of 1.368. These

plants produced large tubers, as observed in ***figure 3.3***, and these plants had grown very tall, with a relative lack of water to smaller plants, causing greater leaf abscission, potentially resulting in a lower plant mass.

3.2.2. Tuber composition

The typical composition of mature cassava tubers is: 70% water, 24% starch, 2% fibre, 1% protein (Tonukari, 2004). The % dry matter value is calculated by dividing the dry tuber weight by the fresh tuber weight. Except for AMM2-52 plants in big pots, the mean % dry matter content (***table 3.2***) for the tubers of all three trials of this study was around the range of 20 – 30% (i.e. 80-70% moisture content). The % dry matter was used to determine the dry tuber weights for ***figures 3.3*** and ***3.4.***

The starch contents of the tubers was significantly lower, than the expected 73.7% and 84.9% dry starch yields (Baguma, 2004), ranging from 7.706% to 40.713%. This is most likely because of the age of the tubers, where they have not swelled with starch, and thus have a greater fibre to starch ratio. Some of the extremely low values are most likely due to misidentification thickened regions of roots as tuberous roots, like the case of SA treated TME3, with a value of 11.921% starch. Amylopectin is the major polymer in cassava, constituting 70% or more, while amylose is the minor, constituting of 30% or less (Taggart & Mitchell, 2009; Wang et al., 2018). In various cultivars, Defloor et al. (1998) found amylose contents of the starch samples varied between 17.9% and 23.6%, for tubers from young plants (9 months of growth). From these results (***table 3.2***) tubers of the transgenic and control lines and cultivars including values, 17.045% to 38.297% with a few outliers, such as SA treated TME3 with a value of 78.224%. This is possibly an effect of misidentification of thickened regions of roots, as tuberous roots, as observed with the starch tests, where the same SA treated TME3 tuber had an outlying low fresh starch content. From these results, there does not seem to be any obvious variations in the composition of the transgenic lines, in comparison with the control cultivars. The treatments of trial 2 (pot size) and 3 (salicylic acid), excluding samples that seemed to be outliers, did not seem to have an effect on any of the compositions mentioned herein either.

Figure 3.2 **Images of frozen tubers harvested from trial 1 (top left), 2 (bottom left), 3 (bottom right) and the plastic box (top right)**

Table 3.2 Mean values of characteristics of tubers collected

Trial	Line/ cultivar	Sub treatment	No. of plants with tubers /No. of plants	Tuber mass (g)	%Dry content	Harvest index	Total starch content (Fresh)%	Amylose%
1	AMM2-52	Infected	0.667[3]	0.670[2](0.156)	23.721[2](3.773)	0.039[2] (0.016)		
	CMM6-6	Infected	0[1]					
	cv.60444	Infected	0[2]					
		Mock	0.500[2]	0.280[2] (0.424)	29.286[2]	0.010[1]		
	dsAC1	Infected	0.333[6]	2.590[3] (1.814)	20.155[3](4.230)	0.188[2] (0.177)	26.107[1]	17.045[1]
		Mock	0.250[4]	0.630[1]	33.333[1]	0.040[1]		
2	AMM2-52	Big pots	0.500[2]	1.530[1]	10.000[1]	0.050[1]		
		Small pots	1.00[1]	0.737[3] (0.45)	20.000[3]	0.362[1]	7.706[1]	26.361[1]
	CMM6-2	Big pots	0.500[2]	2.347[3] (2.022)	30.105[3]	0.857[1]	12.019[1]	37.467[1]
		Small pots	0.500[2]	0.640[1]		0.106[1]		
	CMM6-6	Big pots	0.500[2]	4.310[4] (2.538)	23.296[4]	0.836[1]	30.402[1]	21.344[1]
		Small	0[3]					
	cv.60444	Big pots	0.333[6]	2.584[8] (2.368)	30.077[8] (7.382)	0.792[2] (0.331)	17.286[2] (5.560)	38.297[2] (9.264)
		Small pots	0.333[3]	0.300[1]		0.052[1]		
	dsAC1	Big pots	0.600[5]	4.344[7] (3.934)	26.108[7] (7.210)	0.916[3] (0.443)	24.051[3] (9.1140)	22.200[3] (4.809)
		Small pots	0.0833[12]	0.600[2] (0.509)		0.180[1]		
	TME3	Big pots	1.00[4]	2.853[10] (3.87)	24.900 [10] (4.262)	0.354[4] (0.323)	24.077[2] (8.909)	21.378[2] (2.914)
		Small pots	0.818[11]	3.237[20] (3.112)	18.925[20] (13.722)	1.368[9] (2.320)	24.101[2] (9.175)	29.878[2] (1.666)
3	AMM2-52	Salicylic acid	0.222[9]	3.295[2] (2.029)	28.428	0.398[2] (0.252)	40.713[2]	32.405[2]

					[2] (11.537)		(29.628)	(10.567)
		Water spray	0[10]					
	CMM6-2	Salicylic acid	0[9]					
		Water spray	0.500[4]	2.440[2] (2.475)	21.313 [2] (4.428)	0.372[2] (0.192)	12.058[2] (11.240)	18.960[2] (22.010)
	CMM6-6	Salicylic acid	0[2]					
		Water spray	0.400[10]	2.208[4] (2.829)	27.983 [4] (1.950)	0.239[4] (0.301)	26.525[3] (6.789)	36.303[3] (2.194)
	cv.60444	Salicylic acid	0[7]					
		Water spray	0.200[10]	3.140[2] (3.762)	25.286 [2] (18.081)	0.226[2] (0.256)	37.504[2] (43.613)	23.464[2] (5.805)
	dsAC1	Salicylic acid	0.273[11]	2.763[3] (1.874)	28.456 [3] (2.611)	0.250[3] (0.177)	37.504[3] (10.908)	25.722[3] (8.189)
		Water spray	0.200[10]	1.540[2] (0.453)	26.697 [2] (2.884)	0.120[2] (0.025)	26.513[2] (10.937)	54.204[2] (24.774)
	TME3	Salicylic acid	0.111[9]	1.010[1]	31.111[1]	0.058[1]	11.921[1]	78.224[1]
		Water spray	0[4]					
pb	cv.60444	Infected	1.00[1]	4.150[2] (2.362)	23.516 [2] (2)		19.245[1]	17.747[1]
	dsAC1	Infected	1.00[1]	3.306[5] (6.370)	22.379 [5] (5)		33.852[1]	14.443[1]
	TME3	Mock	1.00[1]	2.770[2] (2.843)	11.978 [2] (2)		8.099[1]	30.518[1]

[N]: Sample size
(sd): Standard deviation
pb: from plants grown in transparent Perspex box

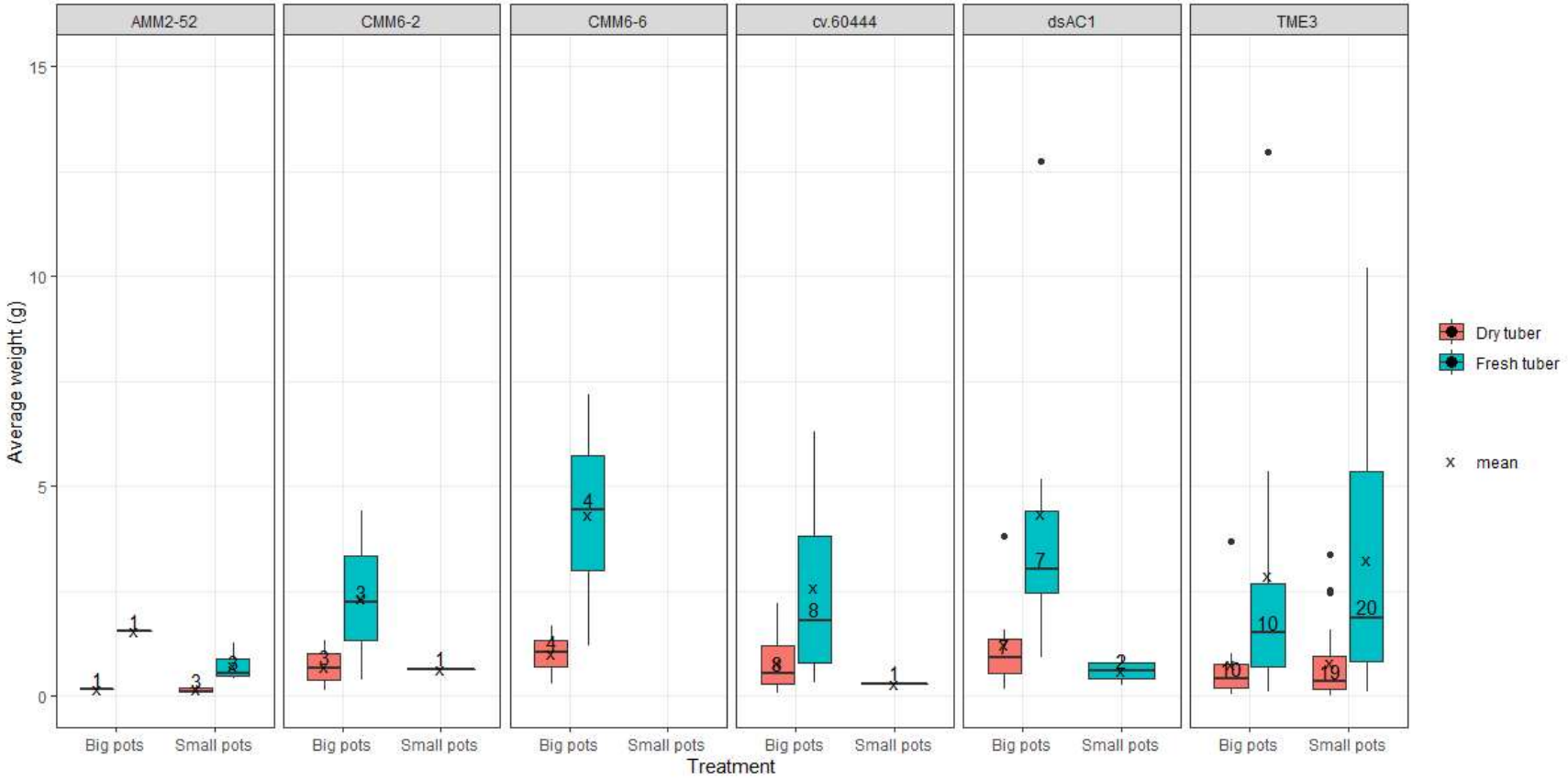

Figure 3.3 **Boxplot of the distribution of dry and fresh tuber weights from trial 2, with the number of tubers weighed**

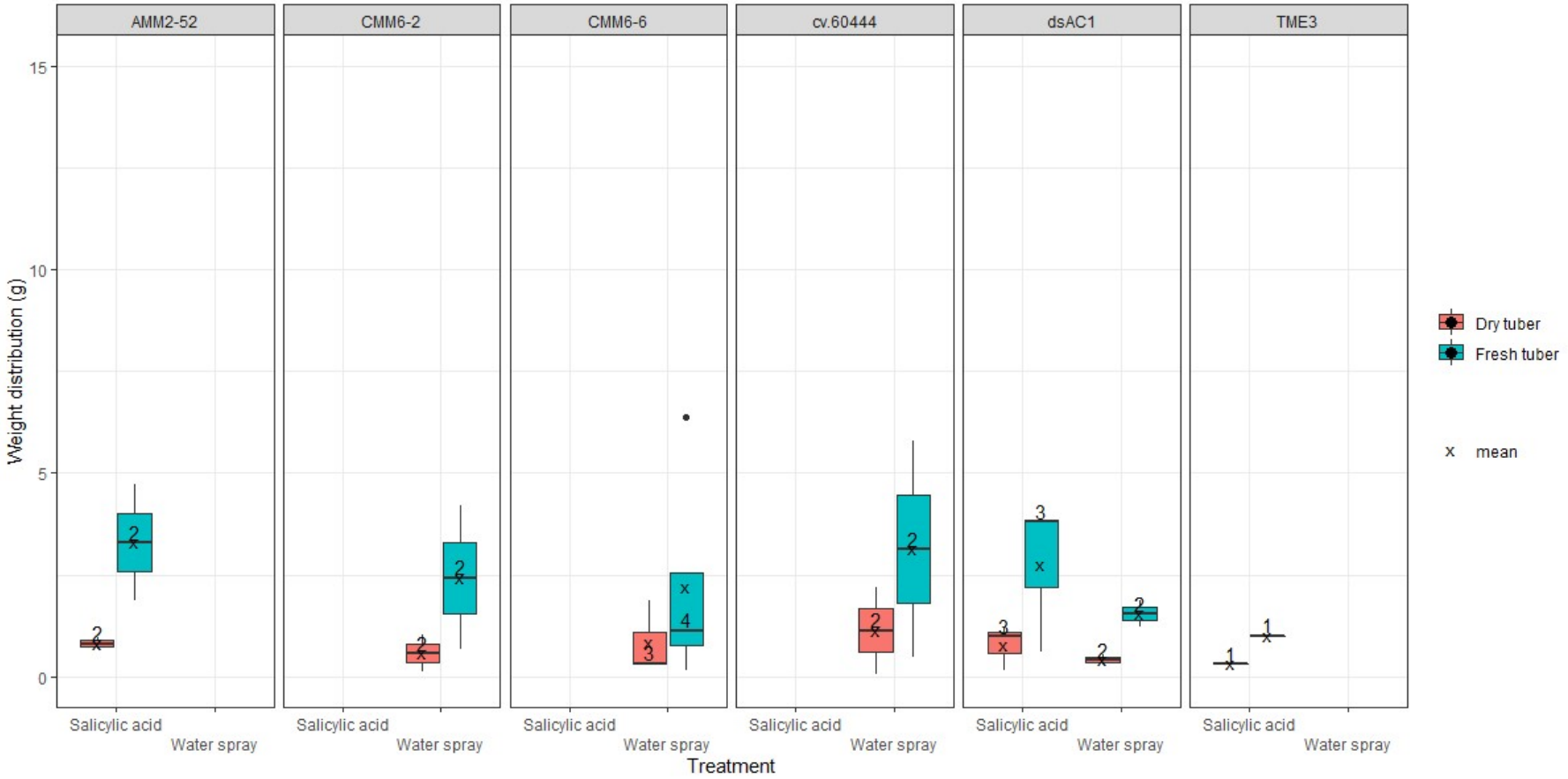

Figure 3.4 **Boxplot of the distribution of dry and fresh tuber weights from trial 3, with the number of tubers weighed**

3.3.3. Pearson correlation

Pearson correlation tests were performed between the characteristics of the tubers and plants, such as leaf number at end of the trial, height at the end of the trial, number of tubers, total starch, and amylose content. The only significant correlation found was between total starch and average tuber mass in trial 2 and 3 (***figure 3.5*** and ***3.6***). The R value was 0.56 for the relationship in trial 2, and 0.8 in trial 3, with significant p values, of 0.031 and 0.0019, respectively. This shows a strong positive correlation, where larger tubers are related to a larger fresh total starch %, and smaller tubers are related to a lower fresh total starch %. This would seem to correspond with previous suggestions that the smaller tubers had a greater fibre ratio to starch, and therefore a lower total starch content.

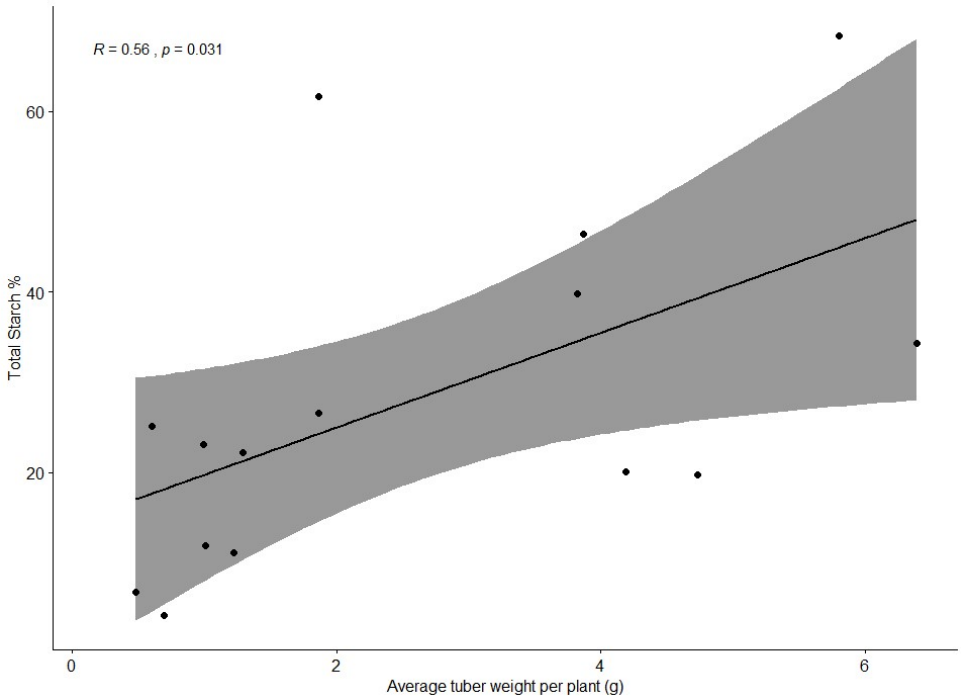

Figure 3.5 **Scatter plot with trend line displaying the significant Pearson correlation between total starch and average tuber weight between the tubers of trial 2**

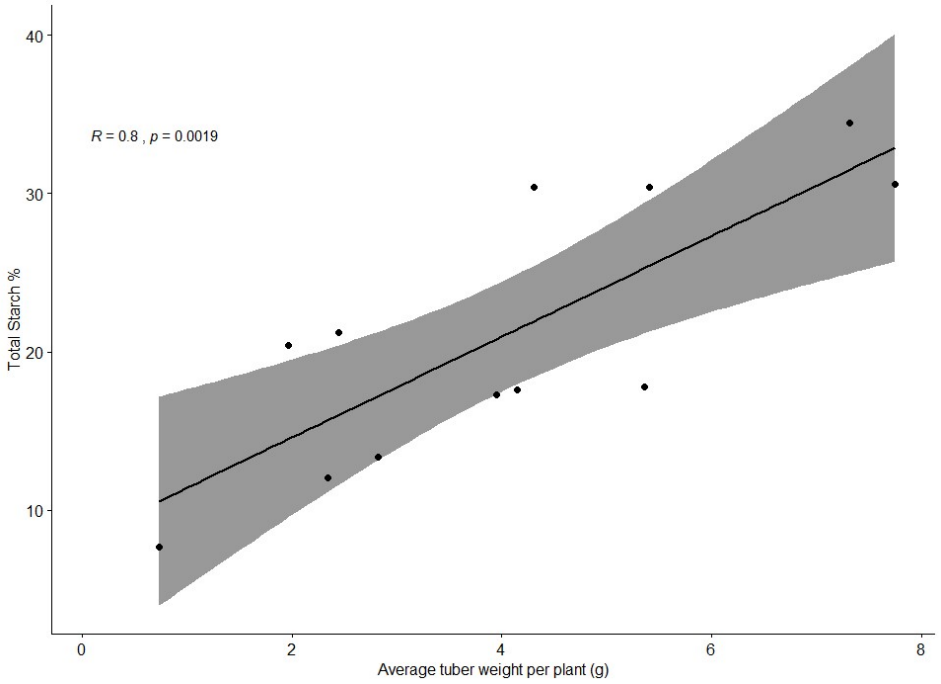

Figure 3.6 **Scatter plot with trend line displaying the significant Pearson correlation between total starch and average tuber weight between the tubers of trial 3**

3.3 Plant characteristics

Pearson correlation tests were performed for height, leaf number, soil moisture and number of stands, or branches, across the period growth. There was a statistically significant positive correlation between height and leaf number in the first three months of growth (***figure S1***). From the fourth month (120 DAP) onwards, this shifted to a statistically significant positive correlation between the number of stands and the number of leaves (***figure S2***). There were no other correlations found between characteristics of the plant growth. It is reasonable to assume that the height of the plant was strongly related to the number of leaves on the plant, up to the point that the plants started branching and producing stands, around 120 DAP. At this timepoint the number of branches/stands becomes a better predictor of the number of leaves, as plants with more branches have more leaves on these branches. This finding is in agreement with leaf area index (LAI) studies that show cassava growth increasing with LAI, up to a point (Cock et al., 1979). This behaviour of branching is likely due to the environment, where the plants are relatively close to the light source, reducing the apical dominance of the plant. As the cassava plants grow taller, they require greater amounts of water and result in greater leaf abscission, and potential stunting if this requirement is not met (Duque & Setter, 2013). There was no correlation found between soil moisture content and leaf count or height in any of the trials.

3.3.1. Least square means

The least square means were calculated for the leaf count and main stem height data, for each line/cultivar, under each treatment, for all three trials. This approach was taken to remediate some of the effects caused by the disparity in sample sizes found between each sub-group (***table 3.3-3.8***). Standard deviation based on the arithmetic mean was added to display the spread of the data. Pairwise t-tests performed at each level (line/cultivar, sub-treatment and their interaction), after performing ANOVA tests. The significant differences found were relatively conservative, due to using Bonferroni adjustment, which reduced type I errors, but might increase type II errors. The t-tests were used to create the grouping superscripts, which indicate where significant differences in the least square means occur. Low sample sizes in some cases trial 1 and trial 2 led to the exclusion of standard deviation and ANOVA for those figures.

In ***table 3.3***, there are no significant differences observed between the means of the groups. This may be a result of the low number of plants, due to mortality after being infected. The AMM2-52 mean height at 180 DAP seems considerably higher than the others, though this may be due to a single plant growing taller than the others, as suggested by the large standard deviation values, and probably does not indicate the line's tolerance to ACMV giving it a growth advantage. Most cassava cultivars usually grow to a maximum of between 1-4 m in height, while the least square means heights are lower than 500 cm, indicating that the plants are still far from reaching their maximum height. ***Table 3.6*** shows a single significant difference between infected and mock values at 60 DAP. This is not likely to represent something, as this trend does not continue at further timepoints. It is interesting to note, that for these two tables, and the other least square means tables (3.3 – 3.8), the standard deviation increases from 90 DAP for both height and leaf number, probably due to a portion of the plants branching.

Table 3.4 represents a larger number of plants, for trial 2, so more significant differences were found. The first is at 60 DAP where plants of the TME3 cultivar are significantly taller than those of cv.60444. This is an expected outcome, as the TME3 cultivar is more robust than cv.60444 and will in general grow taller. At 90 DAP TME3 in big pots is generally significantly taller in big pots than most of the other lines/cultivars, while the TME3 grown in small pots is not. This may indicate the timepoint around when the pot size becomes a bottleneck to the growth of this cultivar. The CMM6-6 line in large pots is also significantly taller than the others, though this may have to do with its low sample size. For the rest, of the trial, plants of the TME3 cultivar were significantly taller than dsAC1 and cv.60444 plants (as they had the greatest sample size). In general, TME3 as a landrace displays a tendency to grow taller than cv.60444, and lines transformed from cv.60444. This is also the only instance where the treatment seemed to have a real impact on the height of the plant. In ***table 3.7***, the means of the number of leaves seem consistent with each other, until the last two months, where TME3 plants from the large pots had more leaves than those from small pots. This could be due to the greater height, or more branches, leading to a greater number of leaves. While this is also a promising result for the recommendation of larger pots, more leaves grown often results in less starch stored in the tubers. This may be supported by the fact that the TME3 plants from the small pots had a larger average mass than those from the large pots (***table 3.2***), despite being shorter, with fewer leaves. In these situations, the water

stress may have contributed to smaller tubers, by causing earlier leaf abscission, and requiring more starch to be appropriated from the tubers, to grow new leaves.

Trial 3 had a far greater number of plants across all lines, leading to more powerful statistical observations. In ***table 3.5***, AMM2-52 started out shorter than the other lines/cultivars, but quickly reached a parity with the other plants, by 60 DAP, with only TME3 plants, on average being taller. TME3 exhibits its tendency to grow tall, by growing taller than cv.60444 for the rest of the trial; and taller than CMM6-2 at the end of the trial. At 120 DAP the salicylic acid (SA) treatment resulted in significant differences in the AMM2-52 and CMM6-2 lines. This is an anomalous result, as the SA has an opposite effect on AMM2-52 to CMM6-2, and this effect is only present at the 120 DAP time point. The results from ***table 3.8*** show a large variety in leaf number throughout the trial. At 0 DAP, the plants start off with fewer AMM2-52 leaves (due to their small size), and fewer TME3 leaves. The AMM2-52 line reaches a parity in number of leaves at 60 DAP, corresponding with its change in height. At 90 DAP, plants from the TME3 cultivar have a greater number of leaves than any of the other lines/cultivars. At 150 and 180 DAP, salicylic acid seemed to have an effect on AMM2-52 and CMM6-6 leaf number, similar to that of its effect on the height of those plants at 120 DAP. SA treatment seemed to have resulted in a greater number of leaves in CMM6-2 plants, with fewer leaves in AMM2-52 plants. There could be a line specific effect of SA, though this is unlikely. It is also suggested that there may be a mild protective effect against water stress, reducing leaf abscission in one line, while lessening the effects of reduced stem growth on the other. It is unknown what effect this would have on tuber production in the long term, if any.

Table 3.3 Least square means of cassava plant height (cm) with standard deviation, measured at intervals of 30 days after potting (DAP) for trial 1

Line/cultivar	Sub-treatments	0 DAP	30 DAP	60 DAP	90 DAP	120 DAP	150 DAP	180 DAP
AMM2-52	Infected	81.33 (22.50) [3][a]	105.00 (24.64) [3] [a]	142.00 (29.14) [3] [a]	170.67 (31.53) [3] [a]	194.00 (66.64) [3] [a]	170.00 (86.62) [3] [a]	344.33 (149.94) [3] [a]
	Mock*	115.00[1]						
CMM6-6	Infected*	102.00[1]	145.00[1]	179.00[1]	190.00[1]	235.00[1]	224.00[1]	402.00[1]
	Mock*	72.00[1]						
cv.60444	Infected	61.75 (25.89) [4] [a]	63.50 (34.70) [4] [a]	120.75 (44.16) [4] [a]	110.50(41.30) [4] [a]	101.25 (67.81) [4] [a]	150.33 (46.05) [3] [a]	184.50 (41.72) [2] [a]
	Mock*	97.00 (28.28) [2] [a]	111.25 (59.75) [2] [a]	189.50 (40.30) [2] [a]	222.50 (82.73) [2] [a]	245.50 (153.44)[2] [a]	235.67 (26.16) [2] [a]	273.50 (129.40) [2] [a]
dsAC1	Infected	59.75 (39.80) [8] [a]	78.14 (47.06) [7] [a]	107.57 (64.93) [7] [a]	149.17 (61.97) [6] [a]	164.16 (60.81) [6] [a]	226.50 (114.88) [6] [a]	273.66 (136.30) [6] [a]
	Mock	73.00 (18.35) [5] [a]	72.40 (16.56) [5] [a]	114.20 (31.38) [5] [a]	149.20 (36.22) [5] [a]	142.00 (29.57) [5] [a]	189.60 (45.39) [5] [a]	264.00 (90.11) [4] [a]

(sd): Standard deviation of plant height
[N]: Number of plants measured
*** Too few samples for t-tests, least square means or sd; mean value and number of plants represented only**

Table 3.4 Least square means of cassava plant height (cm) with standard deviation, measured at intervals of 30 days after potting (DAP) for trial 2

Line/cultivar	Sub-treatments	0 DAP	30 DAP	60 DAP	90 DAP	120 DAP	150 DAP	180 DAP
AMM2-52*	Big pots*	19.00 [2]	58.00 [2]	115.50 [2]	243.00[2]	284.50[2]	366.0 [2]	430.50[2]
	Small pots*	57.00 [1]	103.00 [1]	174.00 [1]	265.00[1]	345.00[1]	399.0 [1]	454.00[1]
CMM6-2	Big pots	101.50 (30.41) [2]^{a}	155.00 (33.94) [2]^{a}	276.50 (28.99) [2]^{ab}	506.00 (9.90) [2]^{ac}	559.00 (5.66) [2]^{ab}	579.5 (41.72) [2]^{ab}	550.00 (127.28) [2]^{ab}
	Small pots	64.50 (33.23) [2]^{a}	117.00 (2.83) [2]^{a}	223.50 (51.62) [2]^{ab}	344.50 (78.49) [2]^{abc}	430.00 (103.24) [2]^{ab}	481.50 (115.26) [2]^{ab}	490.00 (110.31) [2]^{ab}
CMM6-6	Big pots	57.50 (21.92) [2]^{a}	90.00 (59.40) [2]^{a}	170.50 (139.30) [2]^{ab}	232.00 (206.48)[2]^{abc}	270.00 (233.35) [2]^{ab}	361.50 (290.62) [2]^{ab}	398.50 (217.08) [2]^{ab}
	Small pots	37.67 (12.34) [3]^{a}	53.50 (21.92) [3]^{a}	90.50 (36.06) [3]^{ab}	137.50 (123.74) [3]^{abc}	197.50 (152.03) [3]^{ab}	240.00 (197.99) [3]^{ab}	293.00 (176.78) [3]^{ab}
cv.60444	Big pots	47.00 (14.31) [6]^{a}	81.50 (18.65) [6]^{a}	135.50 (48.01) [6]^{a}	207.33 (127.75)[6]^{bc}	274.33 (138.87) [6]^{a}	306.17 (155.20) [6]^{a}	354.00 (171.0) [6]^{a}
	Small pots	33.33 (15.27) [6]^{a}	58.00 (23.94) [4]^{a}	101.00 (30.51) [3]^{a}	194.00 (94.98)[3]^{abc}	235.66 (86.58) [3]^{a}	278.00 (100.96) [3]^{a}	305.00 (89.62) [3]^{a}
dsAC1	Big pots	39.60 (43.44)[5]^{a}	97.40 (64.13) [5]^{a}	165.60 (114.70)[5]^{ab}	266.20 (148.02)[5]^{c}	360.60 (162.25)[5]^{a}	382.80 (192.63) [5]^{a}	423.00 (174.75) [5]^{a}

	Small pots	52.93 (22.88) [15][a]	84.25 (30.29) [12][a]	141.50 (45.84) [12][ab]	195.33(89.41) [12][c]	237.83 (101.58) [12][a]	264.92 (117.10) [12][a]	290.83 (130.13) [12][a]
TME3	Big pots	54.40 (28.17)[5][a]	94.75 (46.60) [4][a]	260.50 (64.38) [4][b]	500.25(68.44) [4][a]	615.75 (82.07)[4][b]	760.25 (78.45) [4][b]	795.50 (174.22) [4][b]
	Small pots	65.09 (36.75) [11][a]	105.73 (62.21) [11][a]	203.54(68.59) [11][b]	305.00(92.24) [11][ab]	392.00 (109.01) [11][b]	487.64 (157.45) [11][b]	553.27 (170.61) [11][b]

(sd): Standard deviation of plant height
[N]: Number of plants measured
Means including different superscripts are significantly different (p < .05), by column with Bonferroni adjustment
*** Too few samples for t-tests, least square means or sd; mean value and number of plants represented only**

Table 3.5 Least square means of cassava plant height (cm) with standard deviation, measured at intervals of 30 days after potting (DAP) for trial 3

Line/ cultivar	Sub-treatments	0 DAP	30 DAP	60 DAP	90 DAP	120 DAP	150 DAP	180 DAP
AMM2-52	Salicylic acid	33.83(11.29) [12]^{a}	45.67(12.73) [11]^{a}	80.18 (14.64) [11]^{a}	161.00 (40.58)[11]^{a}	251.82(73.33) [11]^{abcef}	290.90 (85.69) [10]^{ab}	356.56(92.17) [9]^{ab}
	Water spray	20.75(12.15) [12]^{a}	39.4 (15.16) [11]^{a}	70.00 (29.00) [11]^{a}	114.45 (53.47)[11]^{a}	169.45(79.29) [11]^{abdef}	219.54 (86.80) [11]^{ab}	263.90(84.93) [10]^{ab}
CMM6-2	Salicylic acid	52.25(12.98) [12]^{b}	67.42(23.61) [12]^{b}	86.25 (16.44) [12]^{ab}	130.92 (31.14)[12]^{a}	167.08(28.49) [12]^{abcde}	216.50 (62.28) [10]^{ab}	257.56(76.10) [9]^{a}
	Water spray	58.50(15.09) [12]^{b}	63.00 (9.66) [12]^{b}	108.27 (27.86) [11]^{ab}	153.36 (39.8)[11]^{a}	238.70(59.36) [10]^{abcdf}	259.44 (63.63) [9]^{ab}	216.80(91.90) [4]^{a}
CMM6-6	Salicylic acid	44.25(20.99) [12]^{ab}	57.27 (21.23) [11]^{ab}	82.09 (26.05) [11]^{ab}	136.27 (43.10)[11]^{a}	190.18(74.74) [11]^{abcdef}	224.00 (60.85) [10]^{ab}	230.50(9.19) [2]^{ab}
	Water spray	40.58(20.34) [12]^{ab}	47.25(25.43) [12]^{ab}	74.09 (29.26) [11]^{ab}	137.72 (51.67)[11]^{a}	208.00(81.05) [11]^{abcdef}	257.18 (93.94) [11]^{ab}	314.70(101.26) [10]^{ab}
cv.60444	Salicylic acid	45.83(23.87) [12]^{b}	51.25(28.47) [12]^{ab}	81.58 (44.25) [12]^{ab}	131.91 (53.44)[11]^{a}	162.91(75.74) [11]^{acdef}	191.70 (65.82) [10]^{a}	188.00(49.15) [7]^{a}
	Water spray	46.67(14.58) [12]^{b}	59.83(16.85) [12]^{ab}	89.58 (20.31) [12]^{ab}	142.00 (38.03)[12]^{a}	197.33(67.17) [12]^{acdef}	217.81 (81.32) [11]^{a}	261.50(103.25) [10]^{a}
dsAC1	Salicylic acid	53.67(17.29) [12]^{b}	63.67(13.96) [12]^{b}	88.17 (27.21) [12]^{ab}	146.17 (48.28)[12]^{a}	227.92(90.90) [12]^{abcdef}	278.67(104.31) [12]^{ab}	324.36(121.63) [11]^{ab}
	Water spray	56.17(14.96) [12]^{b}	64.50(22.42) [12]^{b}	81.92(32.07) [12]^{ab}	133.08 (53.80)[12]^{a}	204.25(79.39) [12]^{abcdef}	253.09 (82.69) [11]^{ab}	298.80(94.03) [10]^{ab}
TME3	Salicylic acid	43.08(26.10) [12]^{b}	69.90 (28.24) [10]^{b}	101.50 (31.26) [10]^{b}	195.80 (53.87)[10]^{b}	267.20(89.62) [10]^{bcdef}	317.00 (109.40)[10]^{b}	404.56(95.70) [9]^{b}

Water spray	51.09(18.94) [11]^{b}	64.78 (24.81) [9]^{b}	108.00 (20.72) [8]^{b}	200.50 (24.89)[8]^{b}	269.88(60.85) [8]^{bcdef}	298.86 (46.60) 7]^{b}	378.50(20.50) [4]^{b}

(sd): Standard deviation of plant height
[N]: Number of plants measured
Means including different superscripts are significantly different (p < .05), by column with Bonferroni adjustment

Table 3.6 Least square means of number of cassava leaves with standard deviation, measured at intervals of 30 days after potting (DAP) for trial 1

Line/cultivar	Sub-treatments	0 DAP	30 DAP	60 DAP	90 DAP	120 DAP	150 DAP	180 DAP
AMM2-52	Infected	2.0(0.0) [3]^{a}	3.0(0.0) [3]^{a}	7.7(0.6) [3]^{ab}	10.7(1.2) [3]^{a}	15.3(4.0) [3]^{a}	20.0(6.1) [3]^{a}	22.7(9.0) [3]^{a}
	Mock*	1.0[1]						
CMM6-6	Infected*	4.0[1]	8.0[1]	19.0[1]	19.0[1]	27.0[1]	31.0[1]	22.0[1]
	Mock*	2.0[1]						
cv.60444	Infected	1.8(0.5) [4]^{a}	2.5(0.6) [4]^{a}	6.5(0.6) [4]^{ab}	8.3(1.5) [4]^{a}	7.3(5.9) [4]^{a}	13.0(8.2) [3]^{a}	22.0(11.3) [2]^{a}
	Mock*	4.5(0.7) [2]^{a}	7.5(0.7) [2]^{a}	14.5(2.1) [2]^{ab}	17.5(2.1) [2]^{a}	25.5(0.7) [2]^{a}	35.5(3.5) [2]^{a}	33.0(15.5) [2]^{a}
dsAC1	Infected	2.5(0.9) [8]^{a}	3.3(1.1) [7]^{a}	7.3(2.5) [7]^{a}	10.3(2.1) [6]^{a}	11.0(2.5) [6]^{a}	13.2(4.0) [6]^{a}	16.7(6.9) [6]^{a}
	Mock	2.8(1.3) [5]^{a}	3.4(0.9) [5]^{a}	10.4(1.5) [5]^{b}	13.4(2.5) [5]^{a}	19.0(5.8) [5]^{a}	19.0(9.7) [5]^{a}	18.3(7.5) [4]^{a}

(sd): Standard deviation of number of leaves
[N]: Number of plants measured
Means including different superscripts are significantly different ($p < .05$), by column with Bonferroni adjustment
*** Too few samples for t-tests, least square means or sd; mean value and number of plants represented only**

Table 3.7 Least square means of number of cassava leaves with standard deviation, measured at intervals of 30 days after potting (DAP) for trial 2

Line/cultivar	Sub-treatments	0 DAP	30 DAP	60 DAP	90 DAP	120 DAP	150 DAP
AMM2-52	Big pots*	2.0[2]	5.0[2]	8.5[2]	12.5[2]	14.0[2]	12.0[2]
	Small pots*	5.0[1]	9.0[1]	13.0[1]	16.0[1]	12.0[1]	12.0[1]
CMM6-2	Big pots	6.5(0.7)[2]^{a}	7.5(0.7)[2]^{a}	17.5(0.7)[2]^{a}	16.0(0.0)[2]^{a}	21.5(0.7)[2]^{ab}	25.0(4.2)[2]^{ab}
	Small pots	5.5(2.1)[2]^{a}	9.0(1.4)[2]^{a}	14.5(2.1)[2]^{a}	16.0(4.2)[2]^{a}	19.0(0.0)[2]^{ab}	21.0(0.0)[2]^{ab}
CMM6-6	Big pots	5.5(3.5)[2]^{a}	7.0(4.2)[2]^{a}	14.5(9.2)[2]^{a}	14.5(7.8)[2]^{a}	21.5(10.6)[2]^{ab}	28.0(9.9)[2]^{ab}
	Small pots	3.0(1.0)[3]^{a}	7.0(4.2)[3]^{a}	9.0(1.4)[3]^{a}	13.0(8.5)[3]^{a}	16.0(5.7)[3]^{ab}	15.0(4.2)[3]^{ab}
cv.60444	Big pots	4.7(1.5)[6]^{a}	7.2(1.9)[6]^{a}	11.5(2.2)[6]^{a}	15.8(4.8)[6]^{a}	19.7(5.5)[]^{ab}	23.8(7.5)[6]^{ab}
	Small pots	4.0(1.4)[6]^{a}	6.0(2.1)[4]^{a}	12.3(2.1)[3]^{a}	13.7(1.2)[3]^{a}	16.3(2.1)[3]^{ab}	18.3(0.6)[3]^{ab}
dsAC1	Big pots	5.0(1.9)[5]^{a}	9.0(3.9)[5]^{a}	15.0(6.7)[5]^{a}	15.8(4.9)[5]^{a}	21.2(8.2)[5]^{ab}	28.8(13.3)[5]^{ab}
	Small pots	4.8(1.8)[15]^{a}	9.3(1.8)[12]^{a}	12.9(3.3)[12]^{a}	14.9(3.7)[12]^{a}	14.7(6.7)[12]^{ab}	17.5(7.1)[12]^{ab}
TME3	Big pots	4.2(2.6)[5]^{a}	9.3(1.5)[4]^{a}	12.3(2.2)[4]^{a}	16.8(2.9)[4]^{a}	22.5(2.6)[4]^{a}	31.3(4.9)[4]^{a}
	Small pots	5.0(1.9)[11]^{a}	9.4(3.3)[11]^{a}	11.9(1.0)[11]^{a}	15.5(2.4)[11]^{a}	17.5(2.8)[11]^{b}	18.5(3.4)[11]^{b}

(sd): Standard deviation of number of leaves
[N]: Number of plants measured
Means including different superscripts are significantly different ($p < .05$), by column with Bonferroni adjustment
*** Too few samples for t-tests, least square means or sd; mean value and number of plants represented only**

Table 3.8 Least square means of number of cassava leaves with standard deviation, measured at intervals of 30 days after potting (DAP) for trial 3

Line/cultivar	Sub-treatments	0 DAP	30 DAP	60 DAP	90 DAP	120 DAP	150 DAP	180 DAP
AMM2-52	Salicylic acid	3.6(0.5) [12]^{a}	4.5(0.8) [11]^{a}	8.5(1.1) [11]^{abcd}	10.8(1.5) [11]^{a}	11.6(1.6) [11]^{a}	10.9(3.3) [10]^{acd}	10.9(5.0) [9]^{acef}
	Water spray	2.8(1.0) [12]^{b}	3.7(1.1) [11]^{a}	13.0(1.8) [11]^{abcd}	10.5(3.4) [11]^{a}	12.5(3.0) [11]^{a}	14.9(3.0) [11]^{bcd}	20.1(5.3) [10]^{abdef}
CMM6-2	Salicylic acid	4.1(0.7) [12]^{c}	5.3(1.3) [12]^{b}	17.5(1.8) [12]^{acd}	10.9(2.4) [12]^{a}	13.5(3.7) [12]^{a}	17.9(7.2) [10]^{abc}	24.6(8.3) [9]^{bcde}
	Water spray	3.8(0.6) [12]^{c}	5.4(0.8) [12]^{b}	14.5(0.9) [11]^{acd}	11.4(1.3) [11]^{a}	14.3(2.1) [10]^{a}	11.3(3.5) [9]^{abd}	8.2(7.2) [4]^{abcdf}
CMM6-6	Salicylic acid	4.1(0.8) [12]^{c}	5.2(1.3) [11]^{ab}	14.5(2.1) [11]^{acd}	12.3(2.6) [11]^{a}	14.5(5.1) [11]^{a}	13.9(5.0) [10]^{abcd}	21.0(2.8) [2]^{abcdef}
	Water spray	4.0(1.1) [12]^{c}	5.0(1.5) [12]^{ab}	9.0(1.5) [11]^{acd}	12.1(2.7) [11]^{a}	13.6(2.4) [11]^{a}	9.3(7.0) [11]^{abcd}	13.7(5.9) [10]^{abcdef}
cv.60444	Salicylic acid	4.5(1.2) [12]^{cd}	4.2(1.3) [12]^{b}	11.5(1.7) [12]^{bc}	8.4(3.2) [11]^{a}	12.5(4.6) [11]^{a}	13.1(5.1) [10]^{abcd}	14.3(7.9) [7]^{abcdef}
	Water spray	4.3(1.1) [12]^{cd}	4.5(0.7) [12]^{b}	12.3(1.6) [12]^{bc}	9.4(1.7) [12]^{a}	11.9(2.3) [12]^{a}	12.9(4.4) [11]^{abcd}	16.3(5.6) [10]^{abcdef}
dsAC1	Salicylic acid	4.3(0.8) [12]^{cd}	4.8(0.8) [12]^{ab}	15.0(1.5) [12]^{abc}	11.2(2.7) [12]^{a}	13.8(2.0) [12]^{a}	11.6(7.0) [12]^{abcd}	17.7(9.1) [11]^{abcdef}
	Water spray	4.2(0.9) [12]^{cd}	4.5(1.1) [12]^{ab}	12.9(1.5) [12]^{abc}	11.4(3.2) [12]^{a}	14.8(2.9) [12]^{a}	13.1(5.0) [11]^{abcd}	21.1(5.0) [10]^{abcdef}
TME3	Salicylic acid	3.2(1.0) [12]^{abc}	4.6(0.8) [10]^{ab}	12.3(1.6) [10]^{ad}	13.8(2.7) [10]^{b}	15.5(5.4) [10]^{a}	15.8(8.9) [10]^{abcd}	24.7(11.6) [9]^{abcdef}
	Water spray	3.4(1.2) [11]^{abc}	4.4(1.2) [9]^{ab}	11.9(0.9) [8]^{ad}	13.6(1.8) [8]^{b}	16.1(5.3) [8]^{a}	13.0(5.9) [7]^{abcd}	24.7(16.0) [4]^{abcdef}

(sd): Standard deviation of number of leaves
[N]: Number of plants measured
Means including different superscripts are significantly different ($p < .05$), by column with Bonferroni adjustment

3.3.2. Nonlinear models

After the data was tested for normality with boxplots and Shapiro-Wilk tests, the various nonlinear models were fitted. After the models were fit, their residuals were fitted against time (DAP) and the fit values, as represented in ***figures S3 – S6***. There was no pattern present, apart from heteroscedasticity, which indicates that there were no unpredicted effects. The heteroscedasticity of residuals means that the data for each model became more varied, as each model reached its end time point, leading to greater residuals.

The models chosen for cassava plant height were all sigmoidal in shape, as the growth of most plants over time follows a sigmoidal pattern (Archontoulis & Miguez, 2015). All of the sigmoidal models used have a growth maximum, or y-asymptote, where the growth of the plants plateaus, and they have reached the maximum height in their current environment. The Beta model is an exception, as it can predict a downwards curve, once the maximum growth is reached for the fit data (Yin et al., 2003). With the Beta model, the m parameter was fixed at -60, since the approximate starting point of growth for the plants is known. From ***figures 3.7*** and ***3.8***, all of the models seemed to have fit the height data in a similar manner, with the main differences occurring at the y-axis intercept (starting height when the plants were potted), and the asymptote for the Beta model. Based on the visual fit of the models, only TME3 plants in large pots and CMM6-2 in large and small pots from trial 2 have reached their plateau. The models for CMM6-2 seem to have reached a plateau in trial 3, for the plants with the water spray treatment, but this is likely the result of one of the larger plants dying. In general, growth seems to be greater in plants grown in large pots. From the goodness of fit statistics (***table 3.11***), the logistic model consistently has the lowest Bayesian information criterion (BIC) value, indicating the best fit, in almost all cases. The only exceptions are when the data begins a downward trend after the asymptote, where the Beta model is better at fitting the data, due to its flexibility. The most likely reason that logistic equation fits the data the best, in most cases, is that it has the fewest parameters. This means that the model is less flexible at fitting variations in sigmoidal shapes, and indicates that the other parameters, from the other models, are not necessary for growth during this period.

In ***table 3.11***, the maximum gradient (c_k) is consistent across all models, suggesting that the maximum growth rate has been reached during the six month period of growth. From this, it can be observed that the maximum growth rate is generally higher in plants grown in big pots than small pots, with TME 3 plants in big pots having the greatest maximum growth rate of

7.383 cm/day from the Beta model. There does not seem to be any trend in maximum growth rates between plants treated with salicylic acid or a water control. The predictive power of most of the models is low, but the maximum height can be obtained from the Beta model in a few cases. From this, TME3 in large pots has a maximum height of 801.508 cm, CMM6-2 in large pots has a maximum height of 605.305 cm, and CMM6-2 in small pots has a maximum height of 497.473 cm. These values are lower than the expected maximum height of between 1-4 m (El-Sharkawy, 2003). This means that either the Beta model had not reached the true y-asymptote for growth of these lines/cultivars, or that the restrictive environments of small pots and proximity to the light sources cause a stunting in the height of these plants. It could also be the case that the larger plants experienced a greater water stress, due to absorbing/requiring more water, so their growth was stunted temporarily at this time point. The plants with a vertical height greater than 410 cm would also be reaching the metal shelf above and/or the light source, which could limit growth.

For leaf number modelling, a variety of model shapes were used, as leaf number is a characteristic that is usually not modelled, so there is no established growth pattern. The Monomolecular model used has a shape with an initial growth rate that decreases to a y-asymptote and is sometimes used to model crop yield. From ***figures 3.9*** and ***3.10*** the fitted models have a greater variation in shape. The curve for the Monomolecular model of dsAC1 in big pots is noticeably distorted from the curve that is expected. From the parameter estimates in ***table 3.10***, the k parameter is unsuitable for many cases of Monomolecular and Beta models, with negative values. The Von Bertalanffy model does have appropriate parameters, but the worst fit in most cases, based on the BIC goodness of fit statistics in ***table 3.12***. There does not seem to be any consistent pattern of leaf production from the models chosen, so it is possible that leaf number is not a characteristic appropriate for nonlinear modelling. Crop yield (as leaves) and LAI are characteristics that are often modelled with nonlinear models and should be chosen instead. These may be more suitable characteristics to use for models, by weighing leaf dry mass, or imaging LAI during a study.

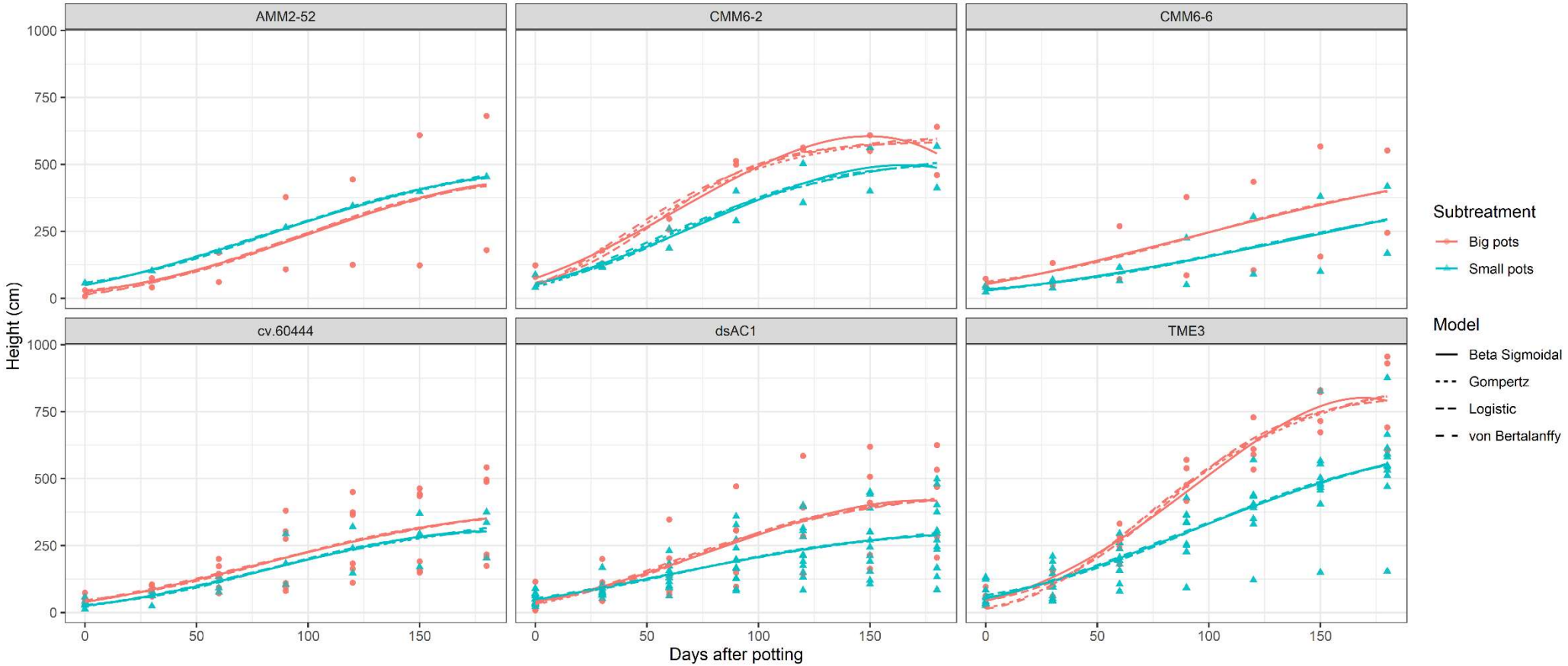

Figure 3.7 **Nonlinear models fitted to plant heights of plants grown in trial 2 with scatterplot of plant height by days after potting**

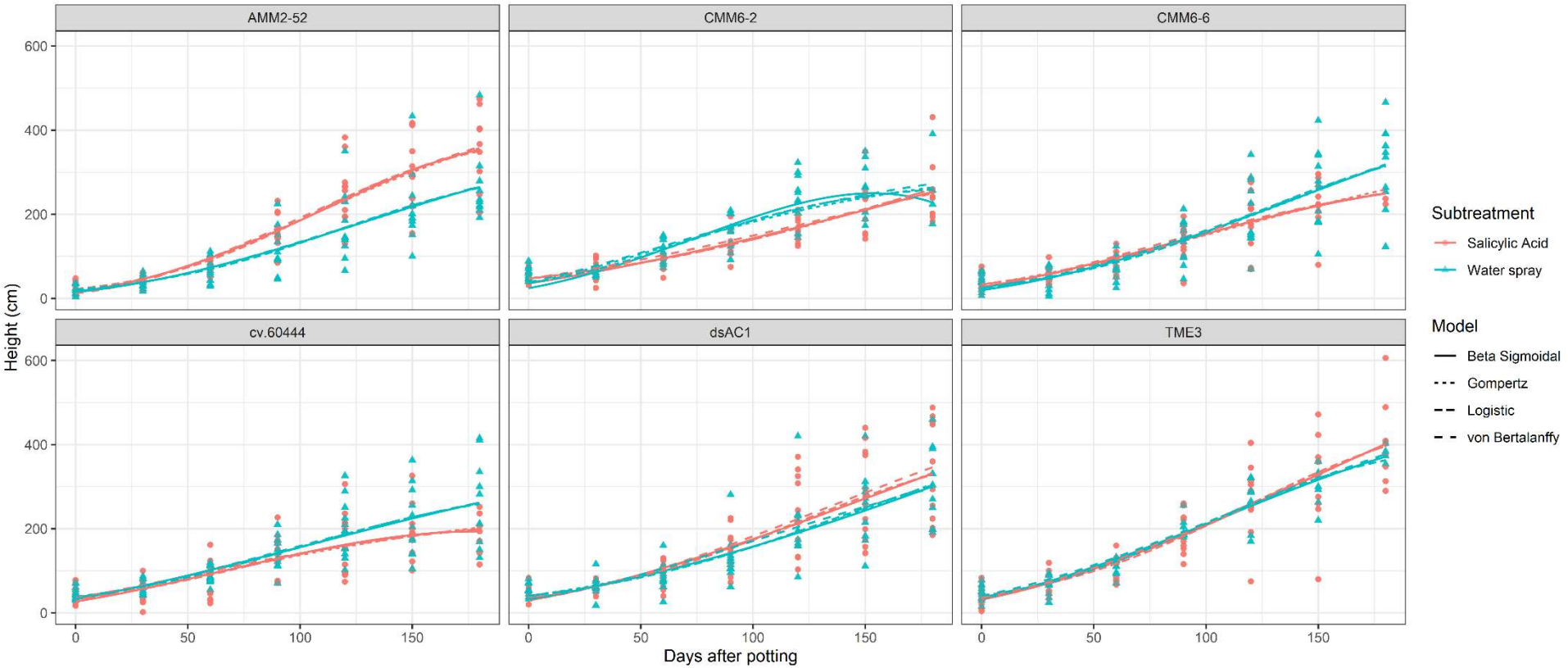

Figure 3.8 **Nonlinear models fitted to plant heights of plants grown in trial 3 with scatterplot of plant height by days after potting**

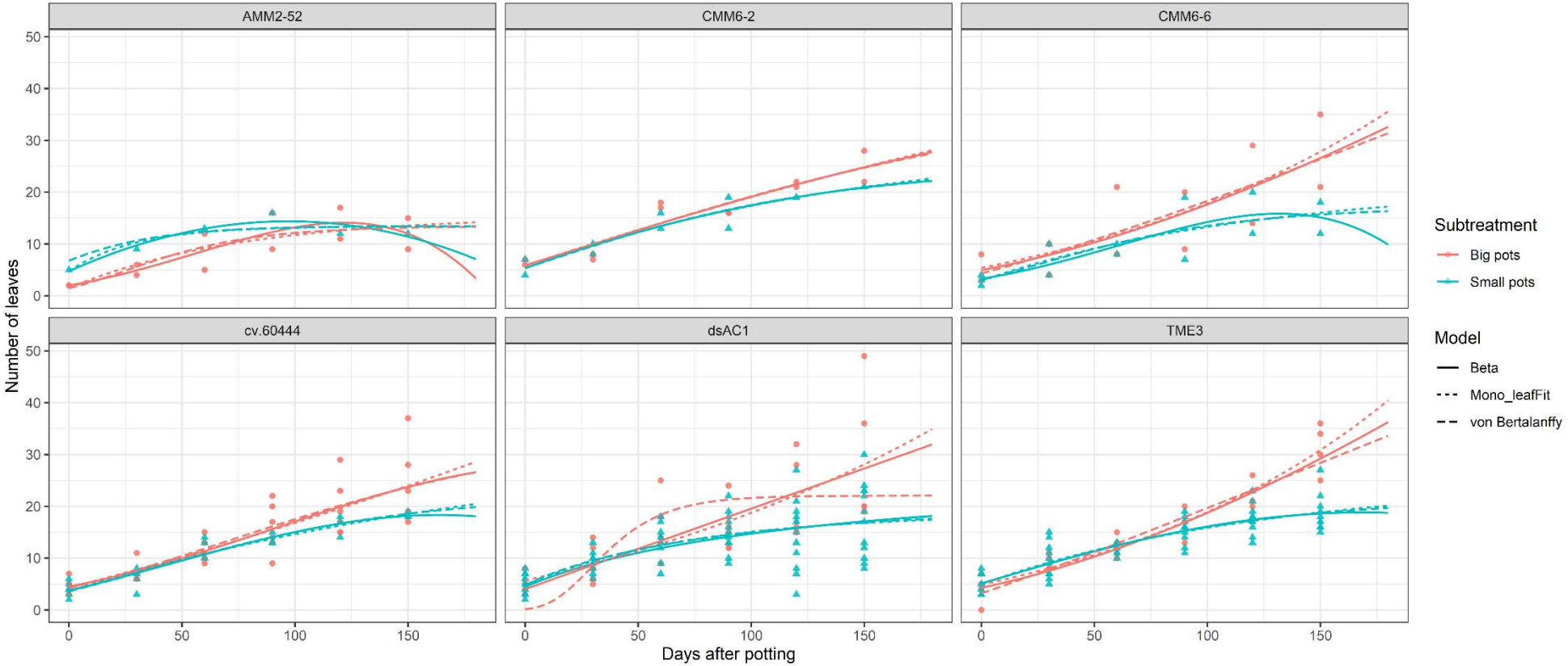

Figure 3.9 **Nonlinear models fitted to number of leaves of plants grown in trial 2 with scatterplot of plant height by days after potting**

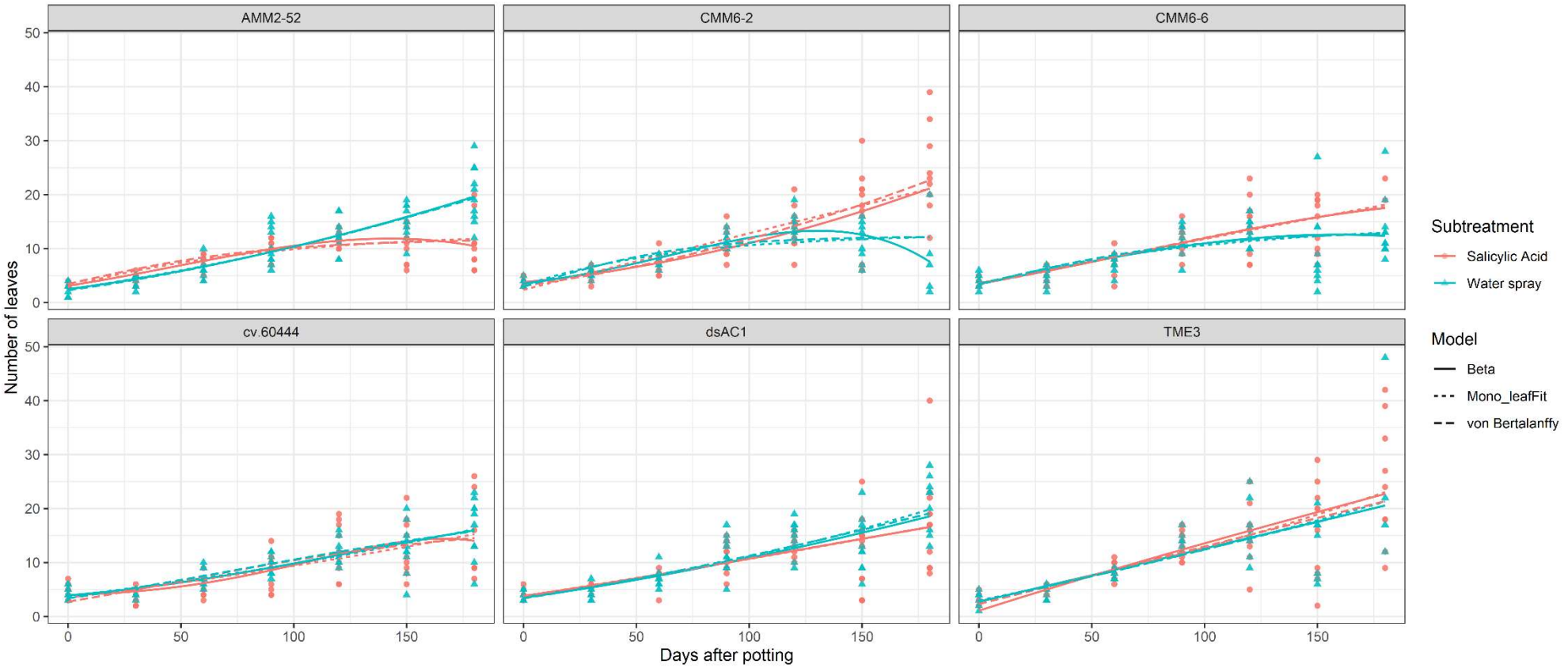

Figure 3.10 **Nonlinear models fitted to number of leaves of plants grown in trial 3 with scatterplot of plant height by days after potting**

Table 3.9 Parameter estimates of the nonlinear models fitted to plant height

Trial	Model	Line/cultivar	Sub-treatment	c_k	α	β	k	m
2	Beta	AMM2-52	Big pots	3.378	435.879	197.437	104.913	-60
			Small pots	3.268	467.593	207.314	84.087	-60
		CMM6-2	Big pots	5.921	605.305	149.257	65.183	-60
			Small pots	4.416	497.473	164.970	73.603	-60
		CMM6-6	Big pots	2.561	485.102	273.349	91.988	-60
			Small pots	2.030	779.436	555.281	218.912	-60
		cv.60444	Big pots	2.532	363.329	207.635	82.453	-60
			Small pots	2.480	303.324	181.435	86.772	-60
		dsAC1	Big pots	3.558	418.696	173.408	80.320	-60
			Small pots	2.160	292.290	196.309	58.259	-60
		TME3	Big pots	7.383	801.508	169.348	94.232	-60
			Small pots	3.916	594.145	221.030	94.894	-60
	Gompertz	AMM2-52	Big pots	2.938	543.134	84.509	0.0147	
			Small pots	2.704	595.998	74.054	0.0123	
		CMM6-2	Big pots	5.410	612.645	39.639	0.0240	
			Small pots	3.755	554.385	51.135	0.0184	
		CMM6-6	Big pots	2.189	597.492	87.672	0.00996	
			Small pots	1.756	630.153	144.201	0.00758	
		cv.60444	Big pots	2.093	460.961	72.711	0.0123	
			Small pots	2.025	378.157	69.890	0.0146	
		dsAC1	Big pots	3.036	483.569	59.080	0.0171	
			Small pots	1.725	347.746	51.177	0.0135	
		TME3	Big pots	6.680	892.771	67.353	0.0203	
			Small pots	3.281	786.967	86.776	0.0113	
	Logistic	AMM2-52	Big pots	3.206	458.437	95.619	0.0280	
			Small pots	2.940	497.288	85.910	0.0236	

		CMM6-2	Big pots	5.846	585.564	55.390	0.0399	
			Small pots	4.012	510.465	67.138	0.0314	
		CMM6-6	Big pots	2.345	474.406	96.294	0.0198	
			Small pots	1.843	381.994	119.580	0.0193	
		cv.60444	Big pots	2.279	384.758	84.552	0.0237	
			Small pots	2.289	320.636	81.399	0.0286	
		dsAC1	Big pots	3.298	435.224	74.169	0.0303	
			Small pots	1.824	307.991	68.057	0.0237	
		TME3	Big pots	7.185	815.604	81.100	0.0352	
			Small pots	3.578	629.606	95.079	0.0227	
	Von Bertalanffy	AMM2-52	Big pots		577.308	-64.569	0.0125	6.225
			Small pots		611.440	-204.099	0.0119	27.217
		CMM6-2	Big pots		616.431	-116.794	0.0242	41.018
			Small pots		558.680	-155.499	0.0176	36.008
		CMM6-6	Big pots		649.429	-129.721	0.00826	5.965
			Small pots		571.586	-234.476	0.00806	18.229
		cv.60444	Big pots		466.484	-226.412	0.0116	31.722
			Small pots		403.604	-204.527	0.0132	39.689
		dsAC1	Big pots		475.298	-170.348	0.0171	47.315
			Small pots		379.849	-241.006	0.0114	29.955
		TME3	Big pots		886.584	-145.794	0.0211	88.508
			Small pots		773.432	-226.141	0.0112	31.942
3	Beta	AMM2-52	Salicylic acid	2.856	364.744	199.972	112.172	-60
			Water spray	1.959	321.405	248.462	128.128	-60
		CMM6-2	Salicylic acid	2.394	7730.092	4687.108	1373.611	-60
			Water spray	2.421	250.012	153.441	73.881	-60
		CMM6-6	Salicylic acid	1.741	266.371	221.546	88.779	-60
			Water spray	2.288	432.328	282.553	139.637	-60
		cv.60444	Salicylic acid	1.563	193.544	178.668	65.957	-60
			Water spray	1.635	343.781	303.267	103.477	-60
		dsAC1	Salicylic acid	2.230	583.822	380.553	160.057	-60

		Water spray	4.096	10605.225	3736.842	1400.866	-60
	TME3	Salicylic acid	2.779	631.062	333.042	150.064	-60
		Water spray	2.527	437.832	252.420	108.514	-60
Gompertz	AMM2-52	Salicylic acid	2.369	532.843	104.130	0.0121	
		Water spray	1.706	490.458	128.110	0.00946	
	CMM6-2	Salicylic acid	2.083	1482.304	324.484	0.00382	
		Water spray	1.592	318.953	57.484	0.0136	
	CMM6-6	Salicylic acid	1.427	431.756	105.422	0.00898	
		Water spray	2.052	683.239	147.081	0.00816	
	cv.60444	Salicylic acid	1.135	258.563	61.767	0.0119	
		Water spray	1.415	438.804	104.230	0.00877	
	dsAC1	Salicylic acid	2.028	756.348	153.046	0.00729	
		Water spray	2.147	1113.660	228.588	0.00524	
	TME3	Salicylic acid	2.461	784.782	133.315	0.00852	
		Water spray	2.171	601.064	104.871	0.00982	
Logistic	AMM2-52	Salicylic acid	2.674	395.464	104.369	0.0270	
		Water spray	1.852	326.154	117.143	0.0227	
	CMM6-2	Salicylic acid	1.559	466.126	162.899	0.0134	
		Water spray	1.786	274.157	71.616	0.0261	
	CMM6-6	Salicylic acid	1.555	290.762	95.186	0.0214	
		Water spray	2.163	400.163	120.272	0.0216	
	cv.60444	Salicylic acid	1.253	215.690	73.938	0.0232	
		Water spray	1.539	311.834	99.702	0.0197	
	dsAC1	Salicylic acid	2.110	425.210	118.490	0.0199	
		Water spray	1.898	447.469	136.156	0.0170	
	TME3	Salicylic acid	2.626	502.688	117.070	0.0209	
		Water spray	2.391	420.863	99.133	0.0227	
Von Bertalanffy	AMM2-52	Salicylic acid		504.399	-176.092	0.0131	35.606
		Water spray		478.468	-241.363	0.00955	32.371
	CMM6-2	Salicylic acid		578.221	-292.431	0.00571	12.008
		Water spray		341.846	-173.540	0.0123	17.326

CMM6-6	Salicylic acid	326.709	-180.621	0.0122	21.408
	Water spray	671.627	-207.048	0.00781	14.904
cv.60444	Salicylic acid	246.221	-167.914	0.0120	13.502
	Water spray	400.309	-231.340	0.00957	21.539
dsAC1	Salicylic acid	705.345	-181.026	0.00767	10.996
	Water spray	496.206	-206.095	0.00890	15.745
TME3	Salicylic acid	769.731	-225.302	0.00816	17.638
	Water spray	655.104	-206.869	0.00859	15.001

α: Maximum value or upper growth asymptote
β: For Gompertz, Logistic – Age (Days after potting) at maximum growth rate, for Von Bertalanffy – Age at growth start, Beta Sigmoidal – Age at the end of growth
k: For Monomolecular – Maximum growth rate, Von Bertalanffy – Growth rate coefficient, Beta Sigmoidal – Age at maximum growth rate
m: For Beta Sigmoidal – Age at growth start, set at -60, Von Bertalanffy – Curve shape parameter
The c parameter for the Beta parameter was removed (set at 0)
c_k: Maximum growth rate from derivative curve

Table 3.10 Parameter estimates of the nonlinear models fitted to number of leaves on each plant

Trial	Model	Line/cultivar	Sub-treatment	α	β	k	m	c
2	Beta	AMM2-52	Big pots	14.108	121.770	56.830	-60	0.223
			Small pots	14.383	96.983	-53.902	-60	-9.976
		CMM6-2	Big pots	35.449	341.574	22.472	-60	-1.014
			Small pots	22.993	229.280	-68.038	-60	-5.645
		CMM6-6	Big pots	215.902	1112.98	455.059	-60	2.062
			Small pots	15.850	131.735	61.099	-60	1.527
		cv.60444	Big pots	27.832	213.595	91.217	-60	2.159
			Small pots	18.338	163.761	43.571	-60	0.034
		dsAC1	Big pots*	7.376×10^{17}	1.074×10^{19}	-3.499×10^{16}	-60	-5.331
			Small pots	21.489	469.137	-10954.24	-60	-268.316
		TME3	Big pots	196.819	905.110	387.915	-60	1.264
			Small pots	18.828	168.809	1.056	-60	-1.430
	Monomolecular	AMM2-52	Big pots	15.494	0.904	0.0131		
			Small pots	13.420	0.647	0.0342		
		CMM6-2	Big pots	89.384	0.935	0.00172		
			Small pots	30.065	0.824	0.00669		
		CMM6-6	Big pots	-8.223	1.654	-0.00650		
			Small pots	21.401	0.865	0.008248		
		cv.60444	Big pots	-58.076	1.074	-0.00183		
			Small pots	33.707	0.892	0.00455		
		dsAC1	Big pots	-15.130	1.375	-0.00488		
			Small pots	18.551	0.742	0.0138		
		TME3	Big pots	-8.042	1.607	-0.00735		
			Small pots	24.976	0.800	0.00786		
	Von Bertalanffy	AMM2-52	Big pots	13.450	-99.957	0.03050	45.573	
			Small pots	13.468	-53.816	0.03391	3.903	
		CMM6-2	Big pots	41.042	-78.295	0.00703	2.261	
			Small pots	25.290	-63.422	0.01221	2.510	

		CMM6-6	Big pots	335.831	-49.711	0.00084	1.363	
			Small pots	17.195	-164.546	0.01938	40.589	
		cv.60444	Big pots	36.389	-232.781	0.01051	23.758	
			Small pots	22.412	-242.783	0.01500	68.197	
		dsAC1	Big pots	22.064	-63.331	0.05477	157.601	
			Small pots	19.794	-13.660	0.00960	0.679	
		TME3	Big pots	261.132	-25.572	0.00093	1.173	
			Small pots	21.622	-83.970	0.01434	4.051	
3	Beta	AMM2-52	Salicylic acid	11.863	142.625	46.702	-60	1.084
			Water spray	6433.957	14939.121	5792.072	-60	0.538
		CMM6-2	Salicylic acid	355.056	1611.717	791.283	-60	2.617
			Water spray	13.317	130.629	65.700	-60	2.473
		CMM6-6	Salicylic acid	18.718	228.622	74.219	-60	1.364
			Water spray	12.598	158.971	13.360	-60	-0.511
		cv.60444	Salicylic acid	14.431	166.523	109.913	-60	3.756
			Water spray	19.237	262.668	125.472	-60	3.028
		dsAC1	Salicylic acid	31.825	536.969	122.151	-60	1.262
			Water spray	452.328	20696.72	6334.347	-60	1.133
		TME3	Salicylic acid	497.951	11851.699	-1768.725	-60	-8.184
			Water spray	37084.190	699906.829	6584.012	-60	-3.111
	Monomolecular	AMM2-52	Salicylic acid	13.044	0.778	0.0117		
			Water spray	-11.981	1.204	-0.00437		
		CMM6-2	Salicylic acid	802.450	0.997	0.000132		
			Water spray	12.794	0.753	0.0149		
		CMM6-6	Salicylic acid	200.080	0.982	0.000429		
			Water spray	14.896	0.779	0.0100		
		cv.60444	Salicylic acid	-18.195	1.191	-0.00241		
			Water spray	-12.085	1.311	-0.00322		
		dsAC1	Salicylic acid	-68.910	1.055	-0.000905		
			Water spray	-10.762	1.336	-0.00420		
		TME3	Salicylic acid	-24.228	1.123	-0.00307		
			Water spray	-44.054	1.067	-0.00183		

Von Bertalanffy	AMM2-52	Salicylic acid	11.814	-110.977	0.02058	11.371
		Water spray	319.309	-68.573	0.00092	1.763
	CMM6-2	Salicylic acid	575.046	-128.525	0.00105	2.522
		Water spray	12.327	-105.375	0.02613	21.546
	CMM6-6	Salicylic acid	24.226	-286.893	0.00996	32.707
		Water spray	13.323	-130.337	0.01904	15.318
	cv.60444	Salicylic acid	17.821	-177.698	0.01197	14.676
		Water spray	24.082	-258.674	0.00827	15.657
	dsAC1	Salicylic acid	38.289	-129.013	0.00424	2.676
		Water spray	250.606	-83.849	0.00086	1.619
	TME3	Salicylic acid	188.837	-23.205	0.00066	1.053
		Water spray	193.073	-37.249	0.00074	1.173

α: Maximum value or upper growth asymptote

β: For Monomolecular – Age (Days after potting) at midpoint (for displacement along x axis), for Von Bertalanffy – Age at growth start, Beta Sigmoidal – Age at the end of growth

k: For Monomolecular – Maximum leaf production, Von Bertalanffy – Leaf production rate coefficient, Beta Sigmoidal – Age at maximum growth rate

m: for Beta Sigmoidal – Age at growth start, set at -60, Von Bertalanffy – Curve shape parameter

c: Number of leaves 0 Days after potting

***Did not converge**

Table 3.11 Goodness of fit statistics of nonlinear models fitted to plant height

Line/cultivar	Sub-treatment	Model	RSS	RMSE	AIC	BIC
AMM2-52	Big pots	Beta	340909.994	156.047	189.135	191.691
		Gompertz	340147.511	155.873	189.103	191.660
		Logistic	341391.193	156.157	189.154	191.711
		Von Bertalanffy	340109.956	155.864	191.102	194.297
	Small pots	Beta	235.979	5.806	52.490	52.274
		Gompertz	200.368	5.350	51.345	51.128
		Logistic	127.315	4.265	48.170	47.954
		Von Bertalanffy	290.828	6.446	55.953	55.682
	Salicylic acid	Beta	221444.691	53.979	829.945	839.268
		Gompertz	221208.202	53.950	829.864	839.187
		Logistic	217266.700	53.467	828.498	837.821
		Von Bertalanffy	222721.244	54.134	832.382	844.036
	Water spray	Beta	244795.254	56.384	847.473	856.848
		Gompertz	244559.539	56.357	847.399	856.774
		Logistic	244108.367	56.305	847.257	856.632
		Von Bertalanffy	244731.375	56.377	849.453	861.172
CMM6-2	Big pots	Beta	33862.783	49.181	156.804	159.361
		Gompertz	44629.775	56.461	160.670	163.226
		Logistic	33935.360	49.234	156.834	159.391
		Von Bertalanffy	46294.918	57.505	163.183	166.378
	Small pots	Beta	46947.255	57.908	161.378	163.935
		Gompertz	48520.604	58.871	161.840	164.396
		Logistic	46434.940	57.592	161.225	163.781
		Von Bertalanffy	49041.311	59.186	163.989	167.185
	Salicylic acid	Beta	116919.368	38.471	808.876	818.354
		Gompertz	113568.010	37.915	806.578	816.056
		Logistic	113103.388	37.838	806.254	815.732
		Von Bertalanffy	117618.848	38.586	811.347	823.194
	Water spray	Beta	180934.739	50.841	756.669	765.663

		Gompertz	193806.439	52.618	761.480	770.474
		Logistic	185289.501	51.449	758.334	767.328
		Von Bertalanffy	195793.034	52.887	764.194	775.436
CMM6-6	Big pots	Beta	253747.639	134.628	185.001	187.557
		Gompertz	253747.210	134.628	185.001	187.557
		Logistic	254083.078	134.717	185.019	187.576
		Von Bertalanffy	253737.318	134.626	187.000	190.196
	Small pots	Beta	111499.968	86.217	184.274	187.106
		Gompertz	111294.057	86.137	184.246	187.079
		Logistic	111159.447	86.085	184.228	187.060
		Von Bertalanffy	111459.995	86.201	186.269	189.809
	Salicylic acid	Beta	131719.320	44.012	715.662	724.540
		Gompertz	130720.610	43.845	715.145	724.023
		Logistic	128060.621	43.396	713.747	722.625
		Von Bertalanffy	133584.838	44.322	718.619	729.716
	Water spray	Beta	302246.489	62.249	873.813	883.240
		Gompertz	300500.624	62.069	873.361	882.788
		Logistic	297746.986	61.784	872.643	882.070
		Von Bertalanffy	301545.176	62.177	875.632	887.415
cv.60444	Big pots	Beta	460962.463	104.763	517.934	524.884
		Gompertz	460730.516	104.737	517.913	524.863
		Logistic	460219.199	104.679	517.866	524.817
		Von Bertalanffy	461153.844	104.785	519.951	528.640
	Small pots	Beta	76393.038	55.279	279.566	284.442
		Gompertz	76596.699	55.352	279.633	284.508
		Logistic	75518.044	54.961	279.278	284.154
		Von Bertalanffy	76865.671	55.449	281.720	287.815
	Salicylic acid	Beta	183028.283	49.400	805.834	815.104
		Gompertz	184006.628	49.532	806.234	815.504
		Logistic	181171.911	49.149	805.069	814.339
		Von Bertalanffy	185703.872	49.760	808.922	820.510
	Water spray	Beta	244583.909	54.950	886.910	896.488

		Gompertz	243366.928	54.814	886.506	896.084
		Logistic	241838.045	54.641	885.996	895.573
		Von Bertalanffy	244038.363	54.889	888.729	900.701
dsAC1	Big pots	Beta	544154.372	124.689	445.133	451.354
		Gompertz	544321.601	124.708	445.144	451.365
		Logistic	542269.746	124.473	445.012	451.233
		Von Bertalanffy	544961.346	124.781	447.185	454.962
	Small pots	Beta	580835.464	81.708	1021.045	1030.908
		Gompertz	580639.754	81.695	1021.015	1030.879
		Logistic	579874.309	81.641	1020.901	1030.764
		Von Bertalanffy	581833.986	81.779	1023.194	1035.524
	Salicylic acid	Beta	413959.615	70.622	950.262	959.938
		Gompertz	410525.779	70.328	949.571	959.246
		Logistic	405928.732	69.934	948.636	958.312
		Von Bertalanffy	417938.873	70.961	953.057	965.151
	Water spray	Beta	283258.390	59.136	898.801	908.379
		Gompertz	279096.958	58.700	897.602	907.180
		Logistic	276337.696	58.409	896.797	906.375
		Von Bertalanffy	287471.317	59.574	901.997	913.969
TME3	Big pots	Beta	184079.716	79.672	344.217	349.687
		Gompertz	180430.724	78.878	343.637	349.106
		Logistic	176897.014	78.102	343.063	348.532
		Von Bertalanffy	181337.103	79.076	345.782	352.619
	Small pots	Beta	846340.823	104.840	942.992	952.367
		Gompertz	845734.192	104.802	942.936	952.312
		Logistic	846186.722	104.831	942.978	952.353
		Von Bertalanffy	846737.813	104.865	945.028	956.747
	Salicylic acid	Beta	312991.900	66.395	805.268	814.319
		Gompertz	311749.065	66.263	804.986	814.036
		Logistic	311620.482	66.250	804.956	814.007
		Von Bertalanffy	312704.880	66.365	807.203	818.516
	Water spray	Beta	68950.212	35.407	556.443	564.472

	Gompertz	67578.476	35.053	555.337	563.367
	Logistic	65739.497	34.573	553.820	561.849
	Von Bertalanffy	68846.215	35.380	558.360	568.396

RSS, Residual sum of squares; RMSE, Root mean square error; AIC, Akaike information criterion; BIC, Bayesian information criterion

Table 3.12 Goodness of fit statistics of nonlinear models fitted to number of leaves on each plant

Line/cultivar	Sub-treatment	Model	RSS	RMSE	AIC	BIC
AMM2-52	Big pots	Beta	87.196	2.696	67.854	70.278
		Monomolecular	102.573	2.924	67.803	69.742
		Von Bertalanffy	97.499	2.850	69.194	71.618
	Small pots	Beta	7.082	1.086	28.022	26.981
		Monomolecular	14.646	1.562	30.382	29.549
		Von Bertalanffy	18.601	1.761	33.816	32.775
	Salicylic acid	Beta	397.994	2.288	351.512	363.166
		Monomolecular	441.310	2.410	357.364	366.687
		Von Bertalanffy	435.992	2.395	358.442	370.096
	Water spray	Beta	636.063	2.874	391.101	402.820
		Monomolecular	636.512	2.875	389.156	398.531
		Von Bertalanffy	637.896	2.878	391.323	403.042
CMM6-2	Big pots	Beta	64.477	2.318	64.231	66.656
		Monomolecular	64.547	2.319	62.244	64.184
		Von Bertalanffy	64.279	2.314	64.194	66.619
	Small pots	Beta	32.917	1.656	56.164	58.588
		Monomolecular	32.926	1.656	54.167	56.106
		Von Bertalanffy	32.726	1.651	56.094	58.518
	Salicylic acid	Beta	1408.908	4.223	461.801	473.648
		Monomolecular	1478.218	4.326	463.595	473.073
		Von Bertalanffy	1334.302	4.110	457.503	469.350
	Water spray	Beta	416.063	2.438	333.415	344.658
		Monomolecular	609.393	2.951	358.129	367.123
		Von Bertalanffy	590.893	2.905	357.971	369.214
CMM6-6	Big pots	Beta	413.906	5.873	86.543	88.968
		Monomolecular	409.101	5.839	84.403	86.343
		Von Bertalanffy	416.671	5.893	86.623	89.048
	Small pots	Beta	147.402	3.367	78.459	81.284
		Monomolecular	153.361	3.435	76.974	79.234

		Von Bertalanffy	151.885	3.418	78.849	81.673
	Salicylic acid	Beta	732.029	3.281	364.565	375.662
		Monomolecular	736.116	3.290	362.943	371.821
		Von Bertalanffy	730.057	3.277	364.381	375.479
	Water spray	Beta	1211.700	3.941	445.314	457.097
		Monomolecular	1225.092	3.963	444.171	453.598
		Von Bertalanffy	1211.366	3.941	445.292	457.076
cv.60444	Big pots	Beta	604.349	4.097	213.706	221.624
		Monomolecular	607.024	4.106	211.865	218.199
		Von Bertalanffy	608.493	4.111	213.952	221.870
	Small pots	Beta	58.797	1.635	94.060	99.515
		Monomolecular	61.123	1.667	92.914	97.278
		Von Bertalanffy	58.313	1.628	93.878	99.334
	Salicylic acid	Beta	1014.404	3.678	418.183	429.771
		Monomolecular	1064.578	3.768	419.804	429.074
		Von Bertalanffy	1086.058	3.805	423.302	434.890
	Water spray	Beta	633.109	2.796	406.420	418.392
		Monomolecular	637.193	2.805	404.941	414.518
		Von Bertalanffy	652.113	2.837	408.815	420.788
dsAC1	Big pots	Beta	1395.253	6.820	210.325	217.331
		Monomolecular	1374.659	6.769	207.879	213.484
		Von Bertalanffy	1887.985	7.933	219.398	226.404
	Small pots	Beta	1429.663	4.366	443.919	455.506
		Monomolecular	1418.361	4.349	441.323	450.593
		Von Bertalanffy	1417.998	4.348	443.304	454.892
	Salicylic acid	Beta	1696.701	4.521	496.005	508.099
		Monomolecular	1697.349	4.522	494.036	503.712
		Von Bertalanffy	1696.628	4.521	496.001	508.095
	Water spray	Beta	945.703	3.417	438.924	450.896
		Monomolecular	920.394	3.371	434.727	444.304
		Von Bertalanffy	926.911	3.383	437.298	449.270
TME3	Big pots	Beta	194.172	2.787	132.194	138.288

	Monomolecular	175.498	2.650	127.666	132.541
	Von Bertalanffy	218.958	2.959	135.197	141.291
Small pots	Beta	421.079	2.526	319.609	330.557
	Monomolecular	423.317	2.533	317.959	326.717
	Von Bertalanffy	422.471	2.530	319.827	330.775
Salicylic acid	Beta	2393.258	5.806	461.248	472.562
	Monomolecular	2334.165	5.734	457.473	466.524
	Von Bertalanffy	2368.649	5.776	460.514	471.828
Water spray	Beta	1524.177	5.264	348.786	358.823
	Monomolecular	1520.340	5.258	346.648	354.677
	Von Bertalanffy	1523.087	5.262	348.747	358.784

RSS, Residual sum of squares; RMSE, Root mean square error; AIC, Akaike information criterion; BIC, Bayesian information criterion

Chapter 4: Conclusions

The starch composition and growth characteristics of the three ACMV tolerant transgenic lines did not display any noticeable irregularities. The starch content of the tubers was justifiably low, due to the young age (six months) of the plants when the tubers were harvested, where smaller tubers correlated with a lower starch content. The mean amylose contents of the harvested tubers mostly fell below the 30% value, as reported in other studies. Starch granule morphology and size was uniform among the transgenic lines and control landraces tested. From these results, we conclude that transformation of cassava cv.60444 with a RNAi hp construct did not affect these tuber characteristics mentioned above, and not unexpectedly, as starch content is related to tuber size. Since the ACMV inoculation killed many of the cassava plants in trial 1, this was not performed in trials 2 and 3. Consequently, it was not possible to determine whether tuber production and characteristics were similar in virus-challenged and mock-inoculated wildtype (untransformed) cv.60444. Virus infection of non-transgenic cassava is expected to lead to a reduction in tuber quantity and quality (Mvududu, 2017). In ACMV-tolerant transgenic lines, tuber production is hypothesised to be affected to a smaller degree, if at all, and be more similar in characteristics and weight to wild-type plants. However, this could not be ascertained in this study and will be performed in future under more optimal light and pot depth conditions.

A method to reliably produce tubers in indoor growth facilities was not successful, but increasing the pot depth seems to be the promising treatment. Bigger pots resulted in a greater chance of tubers being produced and often resulted in an increased growth rate in the cassava plants. Salicylic acid treatment had no strong effects on tuber production, or growth of any of the cassava plants.

Early growth, based on the height of cassava, in controlled environments, can be modelled, with the logistic model having the best fit. The Beta model is the most appropriate model to use if the plants reach their maximum height during their growth. Parameters from the Beta model indicated a lower maximum height of plants grown under the conditions of this trial, compared to what is expected in the field. Cassava has a growing cycle of between nine and 24 months, and the tubers are usually harvested after seven months in the field, depending on the genotype and the environmental conditions. The results of this study were not unexpected, six months seems to be adequate time in growing the tubers, providing the conditions are suitable, however, more parameters need to be tested and more experimental

trials are necessary. Light plays an extremely important role in cassava tuber development, and light averaging 21.99 μmol m^{-2} s^{-1} on the leaf canopy of the plant in the controlled environment may not have been ideal. A previous tuber trial in the greenhouse under natural light proved to be more amenable to tuber production over nine months (Mvududu, 2017). Leaf number is unsuitable for nonlinear modelling, so LAI or yield of leaves should be used instead. In conclusion, a nine month trial in 15 cm pots in a more natural environment such as a greenhouse would be more suitable for tuber production and application of plant modelling as demonstrated in this study.

Chapter 5: References

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Chapter 6: Supplementary data

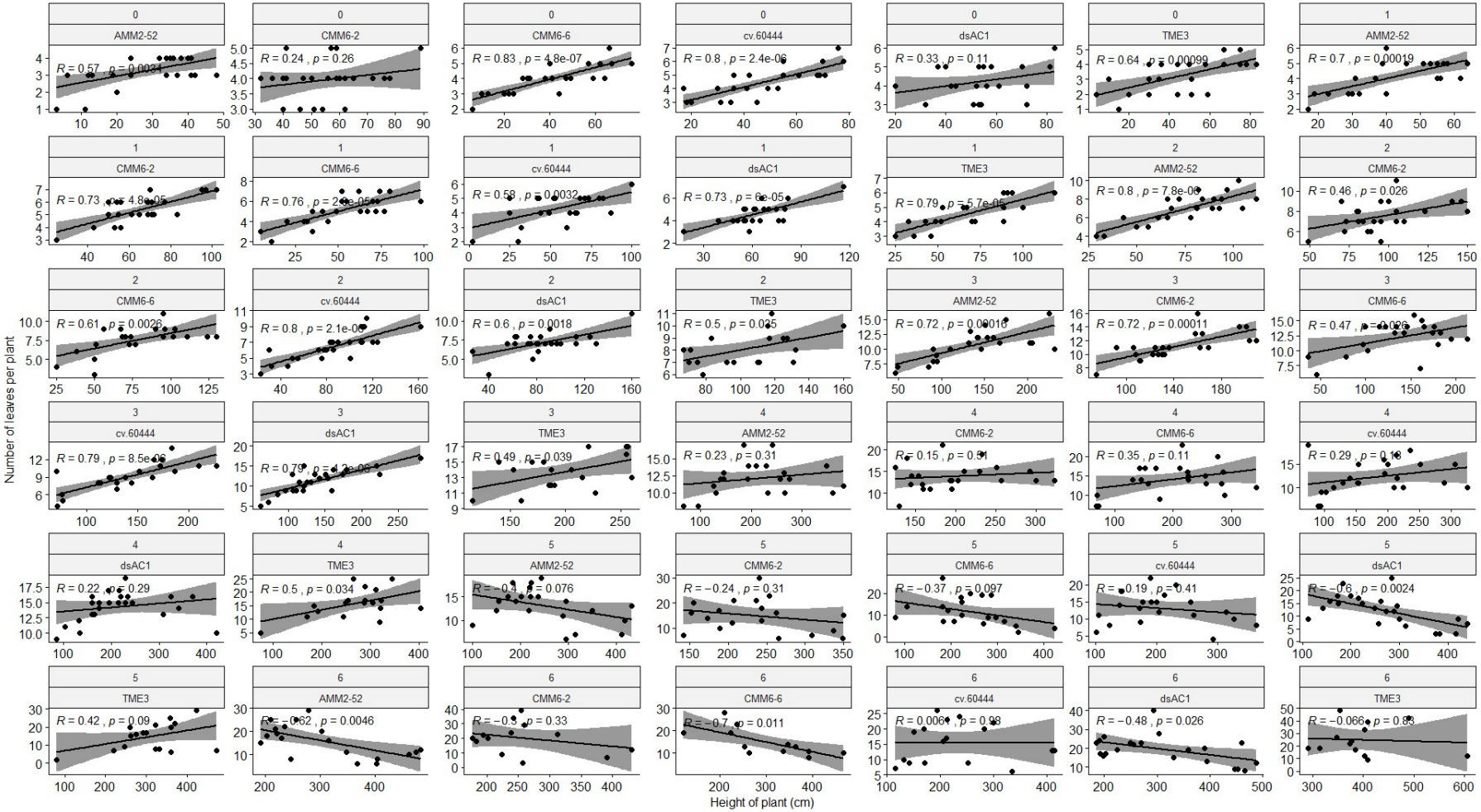

Figure S1 **Scatter plots of height vs number of leaves with Pearson correlation for trial 3 grouped by age and line/cultivar**

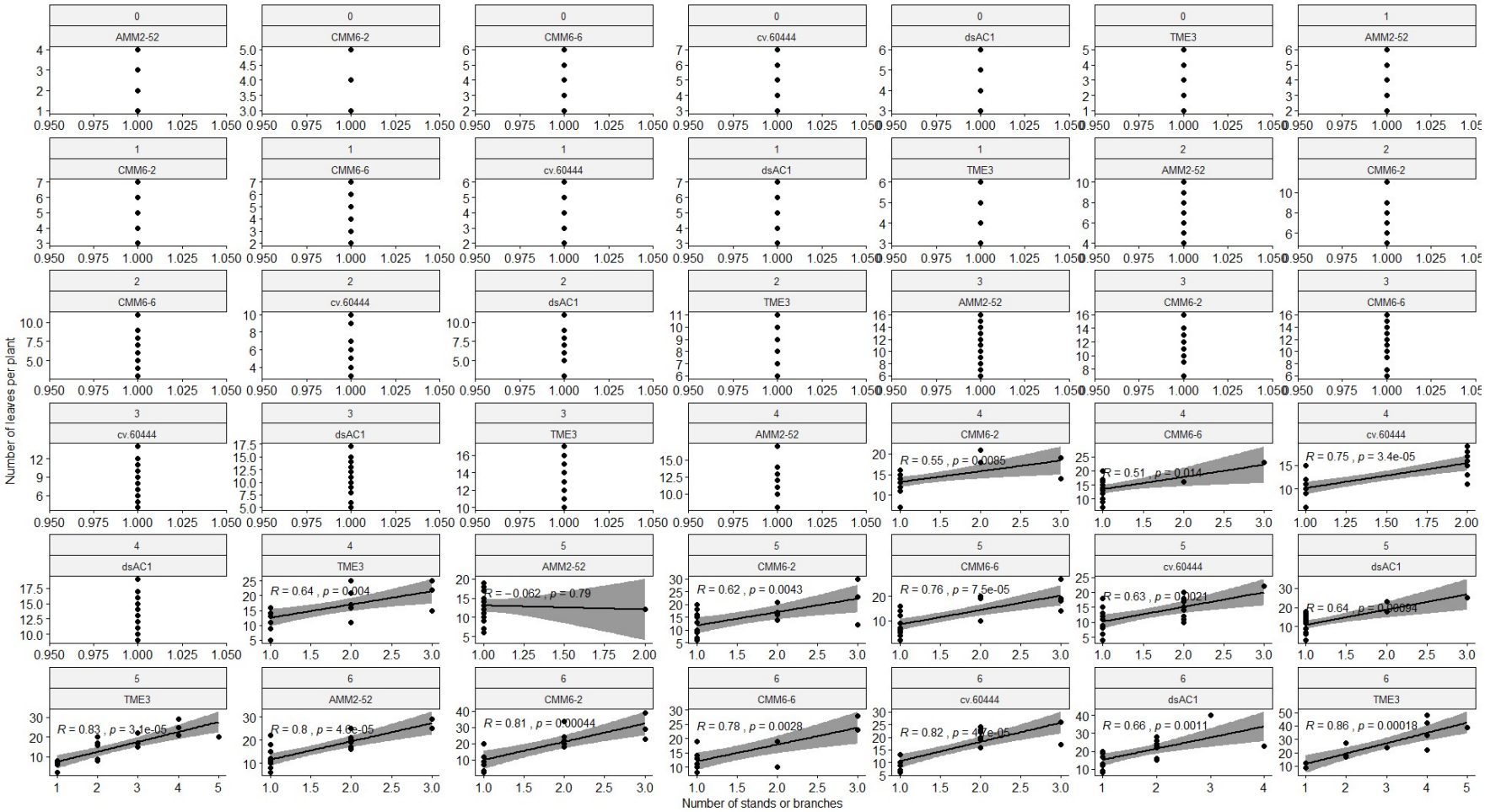

Figure S2 **Scatter plots of number of stands or branches vs number of leaves with Pearson correlation for trial 3 grouped by age and line/cultivar**

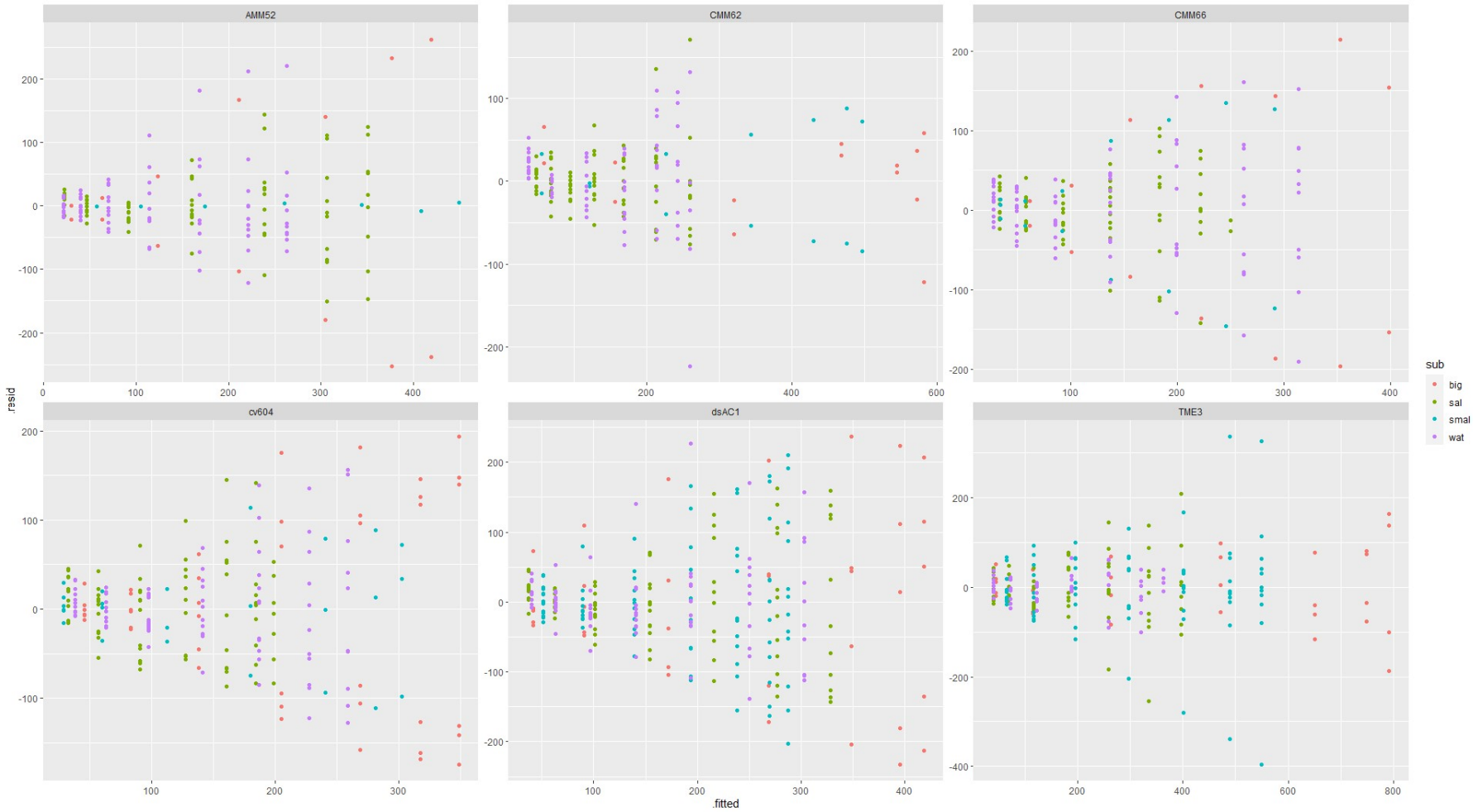

Figure S3 Plotted residuals to fitted values of height from the Logistic model

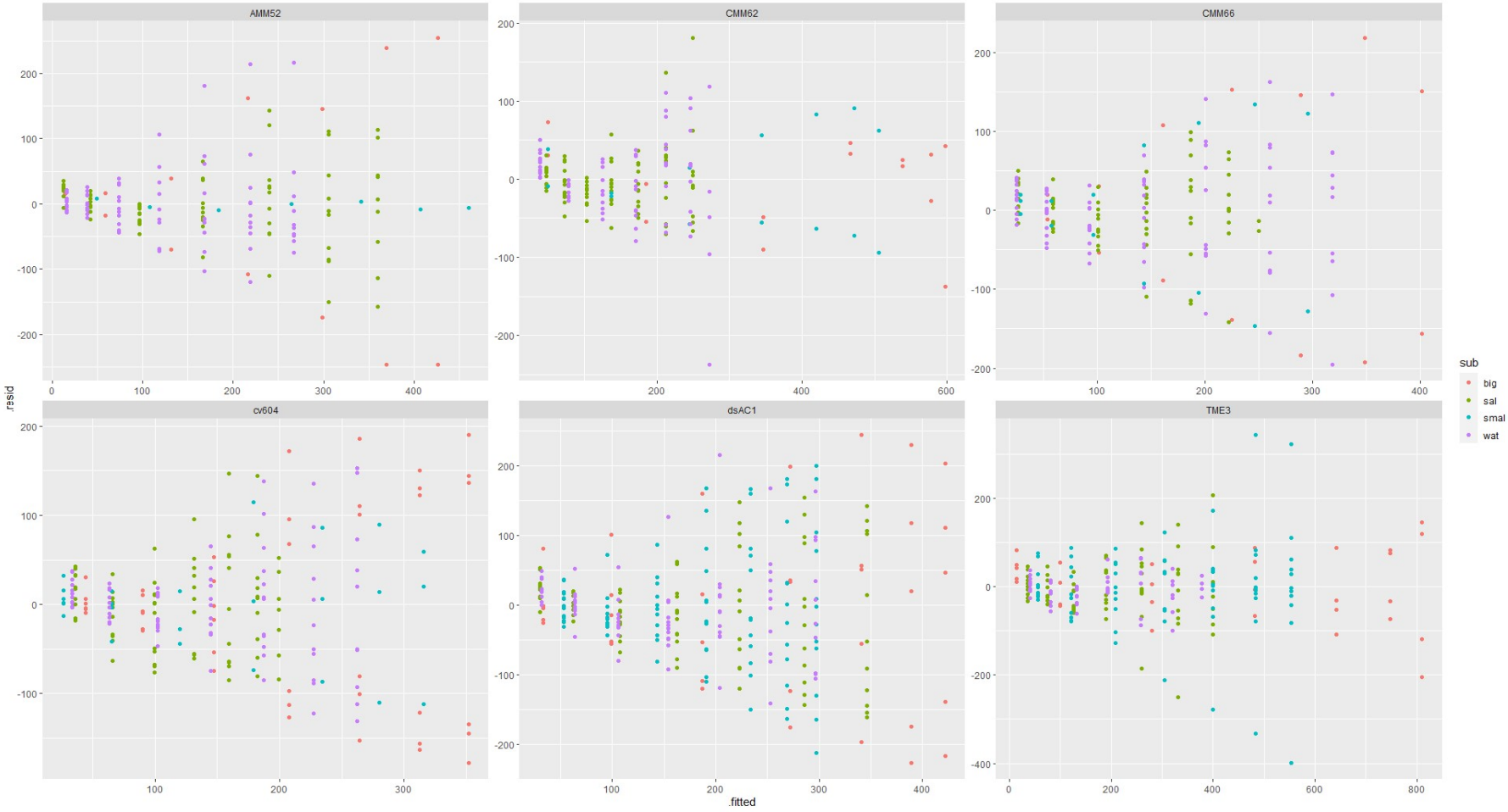

Figure S4 Plotted residuals to fitted values of height from the Von Bertalanffy model

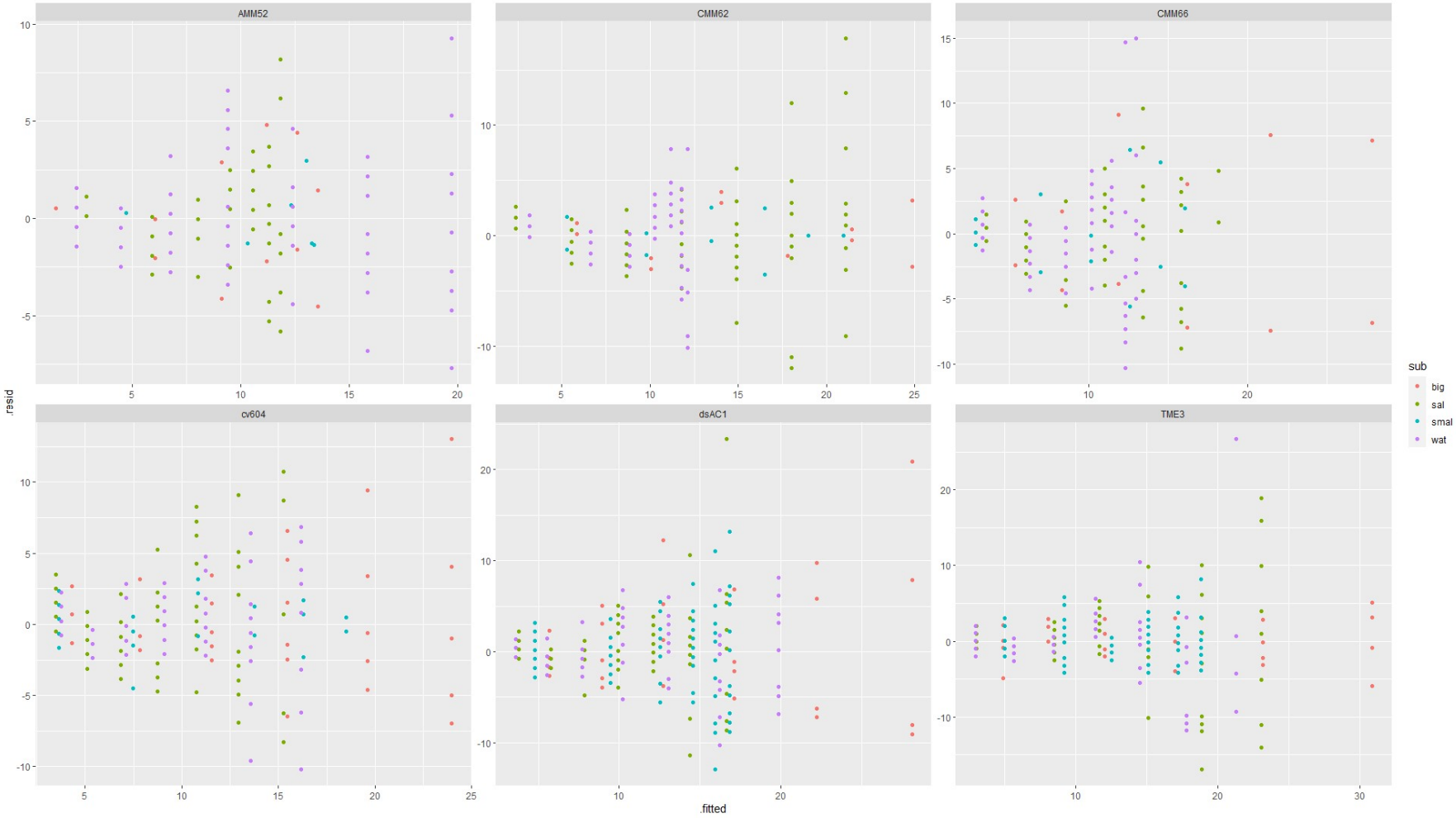

Figure S5 **Plotted residuals to fitted values of leaf number from the Monomolecular model**