UNIVERSITY OF KWAZULU-NATAL

UNIVERSITY OF
KWAZULU-NATAL

INVESTIGATING THE ROLE OF POLYGALACTURONASE IN COTYLEDONAL CRACKING OF GREEN BEANS (*Phaseolus vulgaris* L.)

Thabani Xolani Tshazi

B.Sc (Hons) University of Natal

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

(CROP SCIENCE)

in the

School of Agricultural Sciences and Agribusiness

Faculty of Science and Agriculture

University of KwaZulu-Natal

Pietermaritzburg,

South Africa

2005

ABSTRACT

Transverse cotyledonal cracking (TVC) was characterised at physiological, biochemical and molecular levels in three common bean (*Phaseolus vulgaris* L.) cultivars: Imbali, Tongaat and Tokai. The incidence of TVC was determined visually on ten-day old seedlings and was expressed as the number of cracks per plant. The effect of Ca^{++} on TVC incidence was examined by enhancing the calcium content of seed cotyledons using calcium salts in seed priming and coating. EDAX was used to quantify the cotyledonal calcium content. Activities of the pectinolytic enzymes polygalacturonase (PG) and pectin methylesterase (PME) were assayed at dry seed, VC, R4 and R6 phenophases of the bean plant, and zymogram electrophoresis was used to identify the two enzymes. The results showed that cultivars Imbali, Tongaat, and Tokai were susceptible resistant and intermediate, respectively, with respect to TVC incidence. Calcium uptake was higher in Tongaat cultivar ($P= 0.05$) compared with Imbali and Tokai, irrespective of calcium salt enhancement. Vigour was significantly reduced ($P< 0.01$) by both coating and priming. TVC significantly lowered yield ($P= 0.05$) in the susceptible Imbali cultivar. Zymogram analysis identified lytic bands at ~45kDa (PG gel) and ~30kDa (PME gel). The activity profile of PME was similar for all cultivars. However, PG activity of susceptible Imbali was high in dry seeds and at the R4 and R6 stages of plant development, whereas the more resistant cultivars displayed high activity at the VC stage only. It is concluded that high PG activity at R4 stage is a more reliable determinant of green bean propensity to cotyledonal cracking.

DECLARATION

I hereby declare that the research work reported in this dissertation is the result of my own investigation, except where acknowledged.

.....

Approved by

.....

Dr. Albert T. Modi (Advisor)

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and gratitude to the following persons for their contribution to this study:

Dr. A.T. Modi, School of Agricultural Sciences and Agribusiness, University of KwaZulu-Natal, for hi supervision and guidance throughout the duration of this study. His insight and willingness to go beyond the call of his duty is greatly appreciated.

Dr. M. Beukes, co-supervisor, School of Molecular and Cellular Biosciences, University of KwaZulu-Natal, for the guidance afforded me in the lab-based aspect of this study.

Fellow students in the Schools of ASA and MCBS, especially Julius Oduocho (PhD) and Kershney Naidoo, and also Goodman Zondi (Technician).

Staff at the Centre for Electron Microscopy (UKZN, Pietermaritzburg campus) for their assistance.

The National Research Foundation, for the financial support which made it possible for me to conduct this research.

My family, whose love and support throughout my career has been invaluable, and is a dept that can never be repaid.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

[1] Percentage germinated seeds after five days incubation at 25°C.

LIST OF APPENDICES

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

According to currently accepted taxonomy, green bean (*Phaseolus vulgaris* L.) belongs in the Fabaceae family of legumes and falls under the *Phaseolus* genus. The plant forms an important part of the human diet, providing nutrients such as protein, vitamins and minerals (McCollum, 1992), and is also widely used as animal feed. More recently, in addition to combating malnutrition, the plant's potential in a range of industrial and pharmaceutical processes has been recognised (Morris, 2003) and efforts are currently under way to improve both its nutrient content and its cultivation methods to obtain maximum benefits from the crop.

Phaseolus vulgaris is believed to have been domesticated in the Americas as far back as 6000 B.C. (Dickson, 1991). The plant was introduced to Africa by Portuguese traders in the sixteenth century (Giller *et al.*, 1994) and quickly became established as a crop in many parts of the continent. Today, *P. vulgaris* is the most important legume cultivated in sub-Saharan Africa, with an annual production exceeding two million tons (Pachico, 1989).

The success of the bean plant in Africa and the various other regions where it has been introduced can be attributed mainly to the plant's adaptability to various climatic and soil conditions (Davis, 1997). From the three ancestral gene pools, viz. *P. vulgaris* var. *aborigineus*, *P. coccineus* and *P. polyanthus* (Davis, 1997), a wide variety of *Phaseolus* species has been developed. Today, the CIAT repository lists more than 27,000 entries of bean varieties (http://www. ciat.cgiar.org/urg).

The plant's adaptability to different cropping systems has also endeared it to African farmers with limited access to land. Adaptability has meant the plant can be co-

planted with other essential crops such as maize and tobacco (Pachico, 1989), thus ensuring propagation by the small-scale African farmer.

While *P. vulgaris* is a relatively easy crop to grow, its major production constraint is the legume's susceptibility to a wide variety of diseases. These include both the biotic, such as viral and bacterial infections, and the abiotic, mainly induced by nutrient and mineral constraints (Hall, 1991). In the developing world, where low input systems are employed, such diseases tend to be more severe, and the biotic are often aggravated by abiotic stresses such as mineral deficiency. While better cultivation techniques and the use of nutrient supplements are often the advisable route to follow to minimise the impact on crops in the field, small-scale farmers in the developing world often cannot afford the necessary implements required for modern practice. For this reason, biotechnological approaches to modify crops and equip them with defences against disease provide an important option for disease management.

In this chapter transverse cotyledonal cracking (TVC), a physiological disorder which affects a wide variety of legumes, is discussed. The review of literature was expanded to examine the possible role of the pectin-degrading enzyme, polygalacturonase, in the occurrence of TVC.

1.2 Transverse cotyledonal cracking

Transverse cotyledonal cracking (TVC) is a physiological disorder that affects a wide variety of beans. The phenomenon was first described in the mid-1900s after the work of McCollum (1953) and other pioneering scientists. Prior to this, the cracking of beans was often attributed to improper handling at harvest and efforts were directed at improving machinery and handling. Careful analysis, however, soon showed that some varieties were more prone to cracking than others, suggesting the phenomenon to be heritable. This realisation led to a change in the direction research was taking and attracted the attention of workers in the biological fields.

Transverse cotyledonal cracking is characterised by transverse cracks that appear across the cotyledons of affected plants. The cracks may be observed on both mature quiescent seeds and on the phanerocotylar cotyledons of seedlings. The cracks range in size from microscopic fissures to deep cracks visible to the naked eye (Figure 1.1).

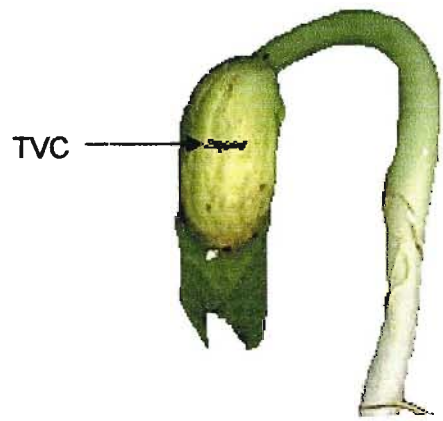


Figure 1.1. Transverse cotyledonal cracking in green bean seedling two days after emergence (Author's specimen).

The incidence of TVC in the field varies widely according to bean variety. In susceptible varieties, the incidence can be as high as 95% (Dickson, 1991), resulting in a reduction in yield by as much as 88% (Eisinger and Bradford, 1986). White-seeded cultivars are generally more susceptible to TVC than darker varieties (Dickson and Boettger, 1977) and resistance is associated with partial dominance (Dickson, 1991).

1.2.1 Impact of TVC on plant development

The main objective of crop farming is to obtain maximum yield from the field. For the bean crop, as with virtually all plants, growth conditions in the early developmental

phases of the seedling largely determine plant stand and, ultimately, yield at maturity. Therefore, to understand the impact of TVC on bean development and yield, it is essential to consider the general development pattern of the bean plant and the role cotyledons play in the relevant developmental stages.

1.2.1.1 Germination and seedling establishment

Germination of green bean involves a series of metabolic processes that transform the quiescent seed into an active, growing entity able to sustain itself. The onset of germination is characterized by rapid imbibition of water through the seed coat. Water uptake is of a triphasic nature commencing with a rapid initial uptake followed by a plateau phase (Bewley, 1997). Further uptake occurs after germination is completed and is accompanied by embryonic axis elongation. The protrusion of the radicle through the seed coat is considered the completion of germination.

Initiation of germination is triggered by external stimuli such as temperature, oxygen and photoperiod. Under optimal conditions of the stimulants, water uptake is initiated and respiration and protein synthesis commence within the seed cells. These metabolic processes are carried out by enzymes and extant mRNAs that survived the desiccation phase that terminates seed maturity (Bewley, 1997). The DNA is repaired at this stage, and new mRNAs are transcribed from which new functional proteins are translated.

The third stage of germination where the rate of water imbibition resumes the exponential curve is in essence a post-germination phase. At the end of the plateau phase, the seed has transcended the dormancy threshold and possesses the necessary molecules to drive metabolic processes for growth into a functional plant. In the third phase DNA synthesis and cell division commence in the embryonic axis resulting in the elongation and protrusion of the radicle. Mobilization of nutrients stored in the cotyledons provides the building blocks needed for radicle elongation (Bewley, 1997). Mobilized nutrients are also translocated to the nascent seedling after radicle protrusion, as the root system at this stage is not sufficiently developed

to sustain the emergent seedling. The nutrients provide building blocks necessary for cell division, wall extension, and establishment of the nascent seedling.

As a stage that demarcates the transition from dependency on the mother plant to an actively growing plant capable of taking up nutrients and developing independently, germination and seedling establishment represent a critical phase where the "normal" unfolding of events is crucial for the overall growth and yield of the legume. Moreover, the translocation of nutrients from the cotyledons to the developing seedling is a vital step that is essential for firm establishment of the seedling. The cracks on TVC-affected cotyledons inhibit the cross-movement of the building blocks needed for growth and development of the seedling, and thus compromise the establishment of the plant at a very sensitive stage. TVC-affected varieties thus have reduced seedling emergence (Aqil and Boe, 1977) and crop yield at maturity (Eisinger and Bradford, 1986).

1.3 Factors affecting transverse cotyledonal cracking

At a physiological level, the cracking of cotyledons results from the tensile stresses that result from water imbibition at germination. Studies from various workers have identified wall strength and rate of water imbibition as the main factors that determine incidence and severity of TVC in affected bean varieties (Dickson, 1973). These traits are in turn determined by factors such as seed coat permeability, cotyledonal nutrient content and seed mineral composition.

1.3.1 Rate of water imbibition

Water movement into the embryo of germinating bean seeds is mainly through the seed coat (testa) tissue, but also occurs through the hilum, micropyle and raphe pores (Agbo *et al.*, 1987; Korban *et al.*, 1981). The thickness and porosity of the seed coat are the major determinants of imbibition rate in the early stages of water absorption, while the sizes of the aforementioned pores play an important role in the

later stages of water absorption (Sefa-Dedeh and Stanley, 1979). Ma *et al.* (2004) also found that the nature of the cuticle deposited on the palisade layer of soybean seed coats determined the permeability to water, with the cuticle of permeable seed coats being mechanically weaker and developing cracks through which water can pass. This finding is in agreement with a previous observation that porous seed coats are usually water permeable while non-porous coats are impermeable (Yaklich *et al.*, 1986).

Initial cotyledonal water and protein composition have also been associated with the rate of water absorption. Cotyledons with a high content of water-soluble proteins tend to have a high solute concentration resulting from solubilisation by innate water retained by the seed after maturation. The high solute concentration creates a low water potential within the cell and thus water uptake is enhanced (Hsu *et al.*, 1983).

Colour is another seed coat characteristic associated with water absorption. Pigmentation in legumes has been shown to decrease the rate of water imbibition (Souza and Marcos, 1993). This phenomenon is thought to arise from the fact that during seed maturation the pigmented seed coats achieve a higher degree of shrinkage than the unpigmented coats, and therefore have greater adherence to the cotyledons – a condition that results in low rates of imbibition (Asiedu and Powell, 1998).

In studies of TVC, rapid water imbibition has been implicated in the occurrence of the cracks. High water-permeability has been linked to high TVC incidence among various cultivars (Dickson *et al.*, 1973). McCollum (1953) found that removal of the seed coat of seemingly resistant cultivars rendered the varieties susceptible to cotyledonal cracking, thus implicating the seed coat as the major factor determining water absorption in the Phaseolus species investigated. McCollum (1953) also found that among susceptible cultivars, seeds with a moisture content of less than 8% at the time of imbibition were more severely affected by TVC than the more hydrated seeds.

1.3.2 Cell wall strength

TVC is accompanied by wall rupture of the affected site brought about by increased tensile stresses resulting from water imbibition. Contrary to McCollum's findings (1953), Morris *et al.* (1970) found that removal of the seed coat and rapid imbibition did not increase the incidence of TVC among resistant cultivars under investigation. This revelation points to possible weaknesses in the structural integrity of walls of TVC -affected cotyledons. While removal of the seed coat may lead to enhanced water imbibition, the integrity of cotyledonary cell walls may be the determining factor whether cracking occurs, and the severity thereof.

As is shown in Figure 1.2, plant cell walls are comprised mainly of cellulosic polymers embedded in a highly hydrated polysaccharide matrix (Cosgrove, 1997). On a dry weight basis, the wall units are mainly 35% cellulose and 30% hemicellulose (the main components of the primary wall), and 35% pectin – the main component of the polysaccharide matrix (Cosgrove, 1997). Structural proteins make up the rest of the dry weight. Cellulose plays a major role in the determination of wall strength and shape while hemicellulose binds the cellulose microfibrils together to make a composite structure (Cosgrove, 1997). The role of pectin in maintaining structural integrity of the wall is to provide the gel matrix in which the cellulose-hemicellulose network is embedded. Pectin is thought to prevent aggregation and collapse of the cellulose network (Jarvis, 1992) and to modulate the porosity of the cell wall to macromolecules (Baron-Epel *et al.*, 1988). Despite the high fluidity of the cell wall (water makes up about two-thirds of plant cell walls; Cosgrove, 1997) the microfibril/matrix network is cohesive enough to withstand the high tensile forces generated by water absorption.

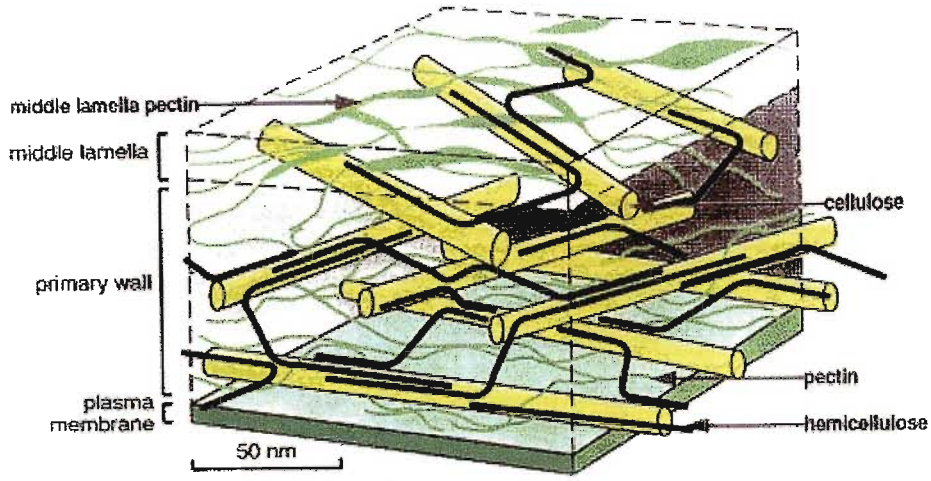


Figure 1.2. Spatial arrangement of the major components of the plant cell wall. [Image from http://www.ippa.info/what_is_pectin.htm]

These networks are kept intact by cross linkages that occur between the side sugars of the sugar polymers comprising the microfibrils (Fry, 1988). The pectin matrix itself is kept in a dense hydrogel state by cross linkages facilitated by (mostly) calcium cations (Marschner, 1995). The crucial role played by calcium in maintaining the structural integrity of walls is evidenced by the high proportion of the element found in the cell walls compared to other cellular structures. Marschner (1995) reported that in apple tissue the cell wall-bound fraction of calcium can account for as much as 90% of the total. A wide range of defects accompanied by collapse of tissue in calcium deficient plants has also been observed. These include the collapse of tissues of organs such as petioles, stems, fruits and seeds from various plant species (Marschner, 1995).

In TVC studies various calcium salts have been shown to limit TVC incidence in the field, and reduce the severity of cracks in the affected cotyledons of sensitive varieties (Dickson *et al.*, 1973; Aqil and Boe, 1975; Dickson and Boettger, 1977; Mazibuko, 2003). The structural role played by calcium in strengthening the walls of TVC sensitive varieties could, in part, account for the apparent resistance observed.

1.4 Potential role of polygalacturonase in TVC occurrence

Despite the viscoelastic and rheological properties of the cell wall as a result of its high fluid content, the bonds between the wall polymers are strong enough to withstand turgor pressure. However, for the plant to grow and develop, wall material has to be incorporated into the existing wall to increase its surface area. This step is facilitated by wall "loosening" enzymes encoded for by the plant (Cosgrove, 1997). Polygalacturonase is one such enzyme that is involved in wall loosening.

Polygalacturonase (PG) genes have been mapped to the genomes of a wide variety of plants. The enzyme is responsible for the degradation of the pectin component of the cell wall. This is required in order to expose the pectin-embedded cellulosic material to hydrolysis by the relevant enzymes. The role played by PG is thus vital for cell growth and differentiation.

Investigations into the metabolic activity of PG have revealed that, as is the case in TVC, various calcium salts have an inhibitory effect on PG activity (Biggs *et al.*, 1997; Cabanne and Doneche, 2002, Flego *et al.*, 1997; Nasser *et al.*, 1999). Moreover, enhanced levels of PG activity are associated with tissue degradation in developmental processes such as fruit softening and organ abscission (Hadfield *et al.* 1998). As a wall-degrading enzyme with an endogenous origin, PG is a viable candidate for the agent responsible for the collapse of cotyledonal tissue in TVC affected plants – a condition that is also heritable.

1.4.1 Plant polygalacturonases

1.4.1.1 Structure and catalytic activity

Molecular characterization of PG has shown it to exist in a number of forms between species, with polymorphism also existing within a species. Regardless of this, steriometric analysis using several methods has revealed what is thought to be the basic structure of a functional PG molecule (Figure 1.3). Moreover, analysis of the

active site of the molecule from different sources has revealed conserved regions thus allowing for a model of the activity of PGs in general to be deduced.

Polygalacturonase, a molecule grouped in the family 28 of glycosyl hydrolases (van Santen *et al.*, 1999), hydrolyses the (1-4) – glycosidic bond between adjacent galacturonic acid residues in homogalacturonan (HG) (Marschner, 1995). The enzyme is a right-handed parallel β-helix molecule (van Santen *et al.*, 1999) with a cleft that serves as the active site (Figure 1.3). Studies of the different PGs isolated from various organisms and species have revealed eight conserved amino acid residues in the active site, *viz.* Asn178, Asp180, Asp201, Asp202, His223, Gly224, Arg256, and Lys258 (van Santen *et al.*, 1999). Together these residues confer an overall negative charge to the active site cleft. The overall negative charge of the HG substrate (Alberts *et al.*, 1983) requires a positively charged region on the active site for binding to take place. This is conferred by two positively charged amino acid residues found at the edge of the negatively charged cleft (van Santen *et al.*, 1999). These molecules bind the exposed R-COO^- group of the substrate.

Catalysis of the HG scissile bond is shown in Figure 1.4. The reaction is co-ordinated by at least two amino acid residues conserved in the active site of PG. One residue acts as a proton donor to the glycosidic oxygen of the scissile bond, while the other activates a water molecule that hydrolyses the reducing anomeric carbon (van Stanten *et al.*, 1999). The reaction causes the barrel-like structure of PG to spread out resulting in breakage of the HG polymer.

1.4.1.2 Impact of PG Activity on Plant Development

Plant polygalacturonases were identified over 40 years ago (Hadfield and Bennett, 1998) and have subsequently been implicated in many developmental processes that require pectin disassembly and cell separation. These processes range from seed germination to vegetative growth and dehiscence. The germination phase is accompanied by, among other processes, cell division and elongation, and radicle

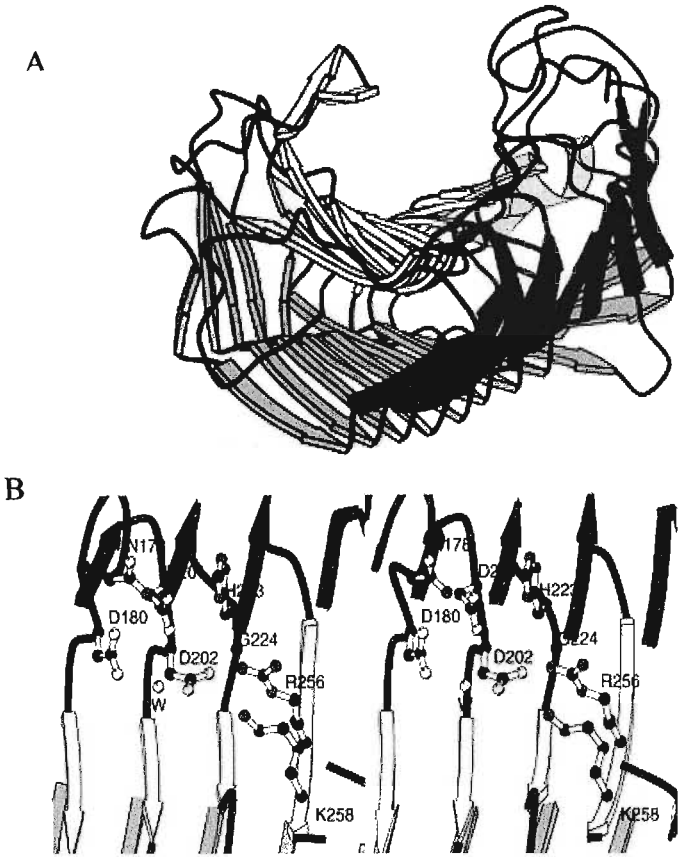


Figure 1.3. A, barrel-like three dimensional structure of polygalacturonase illustrating the active site cleft. B illustrates the sterio view of the active site cleft with the strictly conserved amino acid residues shown in ball-and-stick (van Santen *et al.*, 1999).

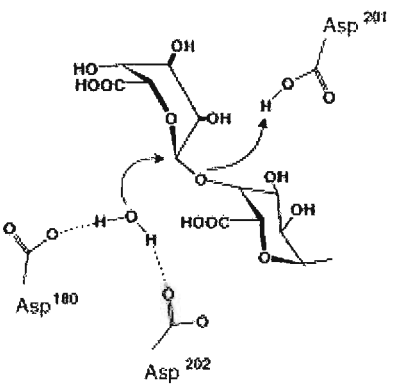


Figure 1.4. Homogalacturonan scissile bond cleavage by polygalacturonase from *Aspergillus niger* (van Santen *et al.*, 1999).

protrusion through the enclosing tissue (Bewley, 1997). As already mentioned, wall loosening is required for the successful incorporation of new wall polymers to increase the surface area for cell elongation. In endospermic seeds such as tomato, PG activity has been implicated in the loosening of the endospermic cap thus allowing the protrusion of the radicle (Sitrit *et al.*, 1999). In embryonic seeds such as beans, PG activity may still be needed for radicle wall loosening to permit turgor-driven elongation of the radicle.

High PG expression and activity has been observed in many structures of seedlings undergoing rapid vegetative growth. Pressey and Avants (1977) detected enhanced PG activity in bean hypocotyls, tomato stems, and roots of beet seedlings. Enhanced PG activity has also been detected in pollen grain maturation and pollen tube growth (Pressey and Reger, 1989; Pressey, 1991), fruit softening (Hadfield *et al.*, 1998), pod and anther dehiscence (Meakin and Roberts, 1991), and organ abscission (Clements and Atkins, 2001).

1.4.1.3 Regulation of PG activity by calcium salts

For activity, polygalacturonase requires unmethylesterified GalA linkages of homogalacturonan. The HG itself is highly methylesterified when deposited to the cell wall, but is de-esterified by pectin methyl esterase (Willats *et al.*, 2001). The un-esterified GalA residues are subsequently cross-linked via calcium ions that bind the exposed $RCOO^-$ groups thus effectively making the recognition sites unavailable to PG (Figure 1.5). In addition to its structural role in wall strengthening, calcium could also directly inhibit PG by virtue of the high affinity the divalent cation has for the negatively charged PG active site. The work of Biggs *et al.* (1997) investigating fungal PG activity *in vitro*, found that a wide range of calcium salts had a significant inhibitory effect on PG activity, with some salts leading to a reduction of 75%. The correlation between calcium concentration and PG inhibition in the presence of an identical carbon source seems to support this theory.

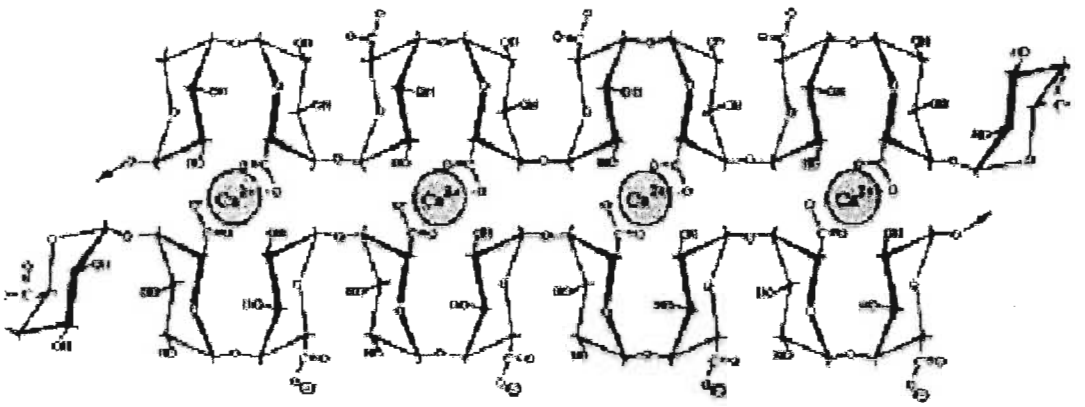


Figure 1.5. Calcium cross linkages (spheres) joining adjacent galacturonic acid molecules. (Carpitan, 1997)

1.5 Conclusion

The preceding review of literature showed that it was previously established that cotyledonal cracking is a significant physiological disorder of leguminous crops and it has been studied in green beans. Recent studies suggested that calcium is important in cotyledonal cracking, but none of them presented conclusive findings about the association of TVC and calcium. Calcium is known to increase the strength of plant cell walls by forming cross-links in the pectin component of the walls. Moreover, calcium has an inhibitory effect on polygalacturonase – an enzyme that hydrolyses homogalacturonic acid in pectin. Based on the evidence, it can be hypothesised that the (hyper-) activity of polygalacturonase is responsible for cotyledonal cracking in affected bean plants.

1.6 Hypothesis and study objectives

The literature reviewed provides circumstantial evidence that polygalacturonase may be important in TVC. This assertion may be justified by several characteristics of PG:

- Studies indicate that TVC is genetic in origin, exhibiting a partial-dominance inheritance pattern (Dickson, 1991). Endogenous

transcripts that weaken plant cell walls are produced by plants in order to facilitate cell elongation and differentiation. PG enzymes form part of the many enzymes produced by plants to this end.

- PG activity has been associated with a variety of tissues undergoing rapid growth in a range of developmental stages. The role played by PGs in these processes is not confined to wall loosening in preparation for elongation by turgor pressure, but PGs are also implicated in tissue degrading processes such as abscission and fruit softening. From germination through to seedling establishment the cotyledons of beans undergo rapid structural changes accompanied by wall modifications to transform the desiccated embryo into a photosynthesising plant. The role for PG in pectin disassembly is therefore palpable and its over-activity could account for the collapse of cotyledonal tissue.

- The inhibition of TVC by calcium salts lends further support to the theory that the over activity PG is responsible for the cracking of cotyledons.

Together, the aforementioned points lend credible, though indirect, evidence that makes PG the prime candidate for a molecular examination of TVC, which was the general objective of this study. Using different *P. vulgaris* cultivars, the study was aimed at:

(i) evaluating the effect of various calcium salts on TVC incidence and

(ii) examining the correlation between PG activity and TVC incidence.

The effect of calcium on bean performance was investigated at growth stages ranging from dehydration after calcium treatment, to post-germination, emergence and seed maturity. Enhanced levels of calcium in the cotyledons after treatment are expected to lower the TVC incidence of each cultivar and thus increase crop output

in the field. The activity of PG is expected to be higher in cultivars with high TVC incidence than those with lower incidence.

References

Agbo GN, Hosfield MA, Uebersax, MA, Komparens K (1987) Seed microstructure and its relationship to water uptake in isogenic lines and a cultivar of dry beans (*Phaseolus vulgaris* L.). Food Microstructure **6**: 91-102

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1983) Molecular Biology of the Cell. Garland Publishing, Inc., New York p 664

Aqil BA, Boe AA (1977) Occurrence of cotyledonal cracking in snap beans and its relation to nutritional status in the seed. HortScience **10**: 509-510

Asiedu EA, Powell AA (1998) Comparisons of the storage potential of cultivars of cowpea (*Vigna unguiculata*) differing in seed coat pigmentation. Seed Science and Technology **26**: 211-221

Barron-Epel O, Gharyal PK, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. Planta **175**: 389-395

Bewley JD (1997) Seed germination and dormancy. The Plant Cell 9: 1055-1066

Biggs AR, El-Kholi MM, El-Nashawy S, Nickerson R (1997) Effects of calcium salts on growth, polygalacturonase activity, and infection of peach fruit by *Monilinia fructicola*. Plant Disease **81**: 399-403

Cabanne C, Doneche B (2002) Purification and characterization of two isozymes of polygalacturonases from *Botyris cinerea*. Effect of calcium ions on polygalacturonase activity. Microbiological Research **157**: 183-189

Carpitan N (1997) Structure and biosynthesis of plant cell walls. In D.T. Dennis, D.H Turnip, DD Lefebvre, eds, Plant Metabolism. Addison Wesley Longman Limited, Singapore, pp 124-127

Clements JC, Atkins CA (2001) Characterization of a non-abscission mutant in *Lunipus angustifolius* L.: Physiological aspects. Annals of Botany **88**: 629-635

Cosgrove, D.J. (1997). Assembly and enlargement of the primary cell wall in plants. Annual Review of Cell Developmental Biology **13**:171-202

Davis JHC (1997) Phaseolus beans. In H.C. Wien, ed, The Physiology of Vegetable Crops. CAB International, pp 409-428

Dickson MH (1991) Transverse cotyledon cracking. In R. Hall, ed, Compendium of Bean Disease. John Wiley & Sons, New York, p 64

Dickson MH, Boettger MA (1977) Inheritance of resistance to mechanical damage and transverse cotyledon cracking in snap beans (*Phaseolus vulgaris* L.). Journal of the American Society for Horticultural Science **102**: 498-501

Dickson MH, Duczmal K, Shannon S (1973) Imbibition rate and seed composition as factors affecting transverse cotyledon cracking in bean (*Phaseolus vulgaris* L.) seed. Journal of the American Society for Horticultural Science **98**: 509-513

Eisinger BA, Bradford KJ (1986) Role of seed mineral content in the occurrence of cotyledon cracking of snap bean. Journal of the American Society for Horticultural Science **111**: 110-114

Flego D, Pirhonen M, Saarilanti H, Palva JK, Palva ET (1997) Control of virulence gene expression by plant calcium in the phytopathogen *Erwinia carotovora*. Molecular Microbiology **25**: 831-838

Fry CF (1988) The Growing Plant Cell: Chemical and Metabolic Analysis. Longman Scientific & Technical, Essex

Giller KE, Anyang BJL, Wilson KJ (1994) The origin and diversity of rhizobia nodulating *Phaseolus vulgaris* in African soils. Advances in Legume Systematics 5: The Nitrogen Factor. In J.I. Sprent and D, Mickey, eds, Royal Botanical Gardens, Kew, pp. 57-62

Hadfield KA, Rose JKC, Yaver DS, Berka RM, Bennett AB (1998) Polygalacturonase gene expression in ripe melon fruit supports a role for polygalacturonase in ripening-associated pectin disassembly. Plant Physiology **117**: 363-373.

Hadfield KA, Bennett AB (1998) Polygalacturonases: Many genes in search of a function. Plant Physiology **117**: 337-343

Hall R (1991) The bean plant. In R. Hall, ed, Compendium of Bean Disease. John Wiley & Sons, New York

Hsu KH, Kim CJ, Wilson LA (1983) Factors affecting water uptake by soybean during soaking. Cereal Chemistry **60**: 208-211

Jarvis MC (1992) Control of thickness of colenchyma cell walls by pectins. Planta **187**: 218-220

Korban SS, Coyne D, Weihing JL (1981) Rate of water uptake and sites of water entry in seeds of different cultivars of dry bean. HortScience **16**: 545-546

Ma F, Cholewa E, Mohamed T., Peterson CA, Gijzen M (2004) Cracks in the palisade cuticle of soybean seed coats correlate with their permeability to water. Annals of Botany **94**: 213-228

Marschner H (1995) Mineral nutrition of higher plants, Ed 2. Academic Press, New York, pp 285-299

Mazibuko TG (2003) Alleviation of cotyledonal cracking in green beans (*Phaseolus vulgaris* L.) by calcium seed treatment. MSc Thesis. University of Natal, Pietermaritzburg, South Africa

McCollum JP (1953) Factors affecting cotyledonal cracking during germination of beans (*Phaseolus vulgaris*). Plant Physiology **27**: 267-274

McCollum JP (1992) Producing vegetable crops, Ed 4. Interstate Publishers Inc., Danville, pp 233-254

Meakin PJ, Roberts JA (1991) Anatomical and biochemical changes associated with the induction of oilseed rape (*Brassica napus*) pod dehiscence by *Dasineura brassicae* (Winn.). Annals of Botany **67**: 193-197

Morris B (2003) Bio-functional legumes with neutraceutical, pharmaceutical and industrial uses. Economic Botany **57**: 254-261

Morris JL, Campbell WF, Pollard LH (1970) Relation of imbibition and drying on cotyledon cracking in snap beans, *Phaseolus vulgaris* L. Journal of the American Society for Horticultural Science **95**: 541-543

Nasser W, Shevchik VE, Hugouvieux-Cotte-Pattat N (1999) Analysis of three clustered polygalacturonase genes in *Erwinia Chrysanthemi* 3977 revealed an anti-repressor functional for the PecS regulator. Molecular Microbiology **34**: 641-650

Pachico D (1989) Bean production problems in the tropics. In H.F. Schwartz and M.A. Pastor-Corrales, eds, Trends in World Common Bean Production, Ed 3. CIAT, Cali, pp 1-8

Pressey R (1991) Polygalacturonase in tree pollens. Phytochemistry **30**: 1753-1755

Pressey R, Reger BJ (1989) Polygalacturonase in pollen from corn and other grasses. Plant Science **59**: 57-62

Pressey R, Avants JK (1977) Occurrence and properties of polygalacturonase in Avena and other plants. Plant Physiology **60**: 548-553

Sefa-Dedeh S, Stanley DW (1979) The relationship of microstructure of cowpeas to water absorption and de-hulling properties. Cereal Chemistry **56**: 379-386

Sitrit Y, Hadfield KA, Bennett AB, Bradford KJ, Downie AB (1999) Expression of a polygalacturonase associated with tomato seed germination. Plant Physiology **121**: 419-428

Souza FHD, Marcos FJ (1993) Physiological characteristics associated with water imbibition by *Calopogonium mucunoides* Des. seeds. Seed Science and Technology **21**: 561-572

van Santen Y, Benen JAE, Schroter KM, Kalk KH, Armand S, Visser J, Dijkstra BW (1999) 1.68Å crystal structure of endopolygalacturonase II from *Aspergillus niger* and identification of active site residues by site- directed mutagenesis. The Journal of Biological Chemistry **274**: 30474-30480

Willarts WGT, Orfila C, Limberg G, Buchholt HC, van Alebeek GJWM, Voragen AGJ, Marcus SE, Christensen MIE, Mikkelsen JD, Murray BS, Knox JP (2001) Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. The Journal of Biological Chemistry **276**: 19404-19413

Yachlich RW, Vigil EL, Wergin W (1986) Pore development and seed coat permeability in soybean. Crop Science **26**: 616-624

CHAPTER 2

DETERMINATION OF THE EFFECT OF CALCIUM SEED TREATMENTS ON TRANSVERSE COTYLEDONAL CRACKING

2.1 Introduction

The performance (yield) of a crop is largely dependent on solid establishment of the nascent seedling in its environment. Establishment is itself dependent on developmental processes from germination to seedling emergence. These developmental processes are facilitated by metabolic reactions that utilize the nutrients stored in the seed and are therefore functions of seed quality. Seed quality and nutrient content thus play vital roles in ensuring vigorous growth and optimal yield of the plant. Complete nutrient availability is therefore essential in maintaining "normal" plant development.

Seed enhancement techniques aimed at improving seed quality by supplementing seeds with the desired minerals have been developed. Seed coating and priming are two methods favoured in seed enhancement. In coating, the seed is enveloped in a water-based polymer (into which the mineral of interest has been added) that forms a hard film after drying (Copeland and McDonald, 1995). While some absorption may occur during application, most of the nutrient uptake occurs after germination when the nascent seedling takes advantage of the high nutrient levels in its immediate vicinity.

Seed priming involves the enhancement of seed mineral content using hydration techniques. Hydration may involve the use of moist solid carriers with high adsorptive capillary forces or solvents with high solute (nutrient) content (Copeland and McDonald, 1995). The dehydrated seeds absorb the nutrient-carrying solution via the diffusion gradient and after dehydration the nutrient is retained in the seed.

Calcium is one of the many elements that serve a crucial role in plant development thus ensuring maximum crop yield. This versatile mineral fulfils a range of structural and physiological functions in plants. As a signalling molecule, calcium plays a role in regulating many physiological processes that influence the plant's response to the growth environment. These processes range from water solute movement, stomatal closure, phosphorylation of the ATPase proton pump, and eliciting of plant defence proteins among others (Marschner, 1995). The divalent calcium molecule also plays an important role in maintaining structural integrity by forming cross-links between wall polymers (Fry, 1988). The critical role played by calcium is underscored by the prevalence of disorders (including tipburn in lettuce, blackheart in celery, blossom end rot in tomato, and bitter pit in apple) in plants grown in calcium-deficient environments (Marschner, 1995). Suboptimal calcium levels have also been observed to impair developmental phases such as germination and root elongation (Marschner, 1995) which in turn compromise early seedling establishment thus reducing plant vigour.

It was previously noted (Mazibuko, 2003) that enhancing the calcium content of bean seeds lowered the incidence of transverse cotyledonal cracking (TVC), a phenomenon that can be attributed to impaired cell wall integrity. The objectives of this exercise were to contrast the efficacy of priming and coating using several calcium salts, and evaluate the effect of cotyledonary calcium content on TVC incidence and yield of the three bean cultivars under investigation.

2.2 Materials and methods

2.2.1 Starting Plant Material

Mature seeds of three *Phaseolus vulgaris* L. cultivars (Imbali, Tongaat, and Tokai) were donated by Pro-seed cc, Pietermaritzburg. The characteristics of starting plant material are presented in Table 2.1. To obtain the moisture content a total of 100

seeds for each cultivar were sampled at random and weighed. The seeds were then dried in an oven at 70°C for 72hrs and re-weighed.

Table 2.1. Seed mass and moisture content of *P. vulgaris* L. cultivars.

Cultivar	Mass[1]	Moisture content ($gH_2O/kgFW$)	Percentage moisture
Imbali	150g	75.55+/-6.18	7.6%
Tongaat	290g	87.89+/-0.26	8.8%
Tokai	200g	85.18+/-1.98	8.5%

2.2.2 Calcium Enhancement

The calcium content of the seeds was enhanced using coating and priming techniques. Seed priming entailed immersing seeds from each cultivar in a Petri dish containing 10ml salt solution for 2hrs, and air drying (room temperature) for 48hrs. In seed coating the 20 seeds were swirled in a Petri dish containing enough drops of the salt-polymer to cover each seed uniformly. Six replicates of 20 seeds each cultivar were treated in 10mM $Ca(NO_3)_2$, 10mM $CaCl_2$, 10mM $CaSO_4$, 100mM $Ca(NO_3)_2$, 100mM $CaCl_2$, and 100mM $CaSO_4$ salt concentrations separately. Seeds immersed in distilled water and those coated with a salt-less primer were treated as controls for priming and coating experiments respectively.

2.2.3 Cotyledonal calcium content

Thin cross-sections of cotyledon tissue from three randomly selected seeds (from each treatment) were used for the determination of calcium content. Calcium content was evaluated before salt treatment, and after drying post-treatment. The environmental scanning electron microscope (ESEM) (Philips XL30) with the energy

[1] Mass per 1000 seeds.

dispersive atomic X-ray analysis (EDAX) program was used to analyze the tissue calcium content. Specimens were analysed at low vacuum mode (1torr) and area scanning was allowed for 100 livetimesecs per reading. The quantitative microanalysis facet of EDAX was used to quantify the calcium content of the scanned area (Modi *et al.* 2004). At least five area readings were taken for each treatment and data in the results are presented as means (± standard error of the mean).

2.2.4 Seed vigour assessment

The percentage of seed germination, seedling abnormality and yield at harvest were all parameters evaluated in order to assess seed quality and seedling vigour after calcium treatment. These were also contrasted with TVC incidence in order to assess whether cotyledonal cracking had a significant impact on the respective variables.

For the evaluation of germination propensity, four replicates of 50 randomly sampled seeds from each treatment were germinated in paper towels dampened with distilled water and incubated at 25°C in a germination chamber. Radicle protrusion of at least 2mm after five days incubation was considered as indicating the completion of successful germination. After eight days the seedlings growing in the germination chamber were evaluated according to the guidelines set by the International Seed Testing Association (AOSA, 1992). The scoring of cotyledons with cracks was done visually with the unaided eye after ten days incubation.

2.2.5 Field trial

In order to simulate growth conditions the three *P. vulgaris* cultivars were grown in an open field at Ukulinga farm, Pietermaritzburg, in the summer of 2004. A randomised complete block design was used and the experiment was replicated three times (see Appendix 1). The experiment was undertaken using a plot that had

lain fallow for 12 months and supplemented with fertilizer to attain 40kg/ha nitrogen, 60kg/ha phosphorus, and 40kg/ha potassium, according to recommendations from soil analysis from Soil Fertility and Analytical Services, Pietermaritzburg, KwaZulu-Natal. Thirteen plots were allocated to each cultivar, with ten seeds per treatment planted per row. The 13 plots corresponded to the different treatments each cultivar received (treatments are shown in Table 2.2. Appendix 1 shows all treatments and field layout). The scoring of cotyledons with cracks was done visually with the unaided eye ten days after planting, and crop yield was evaluated by weighing the seeds at harvest.

2.2.6 Statistical analysis

Analysis of variance was analysed using the GenStat Release 8.1 (PC/ Windows 2000) program. All analysis were carried out at $p = 0.05$. Correlation and regression analysis were performed using Microsoft Excel.

Table 2.2. Treatment structure for seed calcium enhancement.

Cultivar[2]	Treatment	Salt	Concentration
Tokai	distH_2O	-	-
Tokai	Primed	$Ca(NO_3)_2$	10mM
Tokai	Primed	$Ca(NO_3)_2$	100mM
Tokai	Primed	$CaCl_2$	10mM
Tokai	Primed	$CaCl_2$	100mM
Tokai	Primed	$CaSO_4$	10mM
Tokai	Primed	$CaSO_4$	100mM
Tokai	Coated	$Ca(NO_3)_2$	10mM
Tokai	Coated	$Ca(NO_3)_2$	100mM
Tokai	Coated	$CaCl_2$	10mM
Tokai	Coated	$CaCl_2$	100mM
Tokai	Coated	$CaSO_4$	10mM
Tokai	Coated	$CaSO_4$	100mM

[2] The Imbali and Tongaat cultivars were treated similarly.

2.3 Results

2.3.1 Cotyledonal calcium enhancement

Representative ESEM images of the cotyledon tissue analysed are shown in Figures. 2.1 (A) and 2.2 (A), and EDAX spectra are shown in Figures 2.1 (B) and 2.2 (B). Area readings are indicated by the blocks. Sample tissue with thicker, more prominent cell walls registered a calcium peak (Figure 2.1) whereas the fragile walls of tissue in Figure 2.2 show no peak. This observation lends support to the contention that calcium plays a vital role in maintaining wall integrity (Fry, 1988).

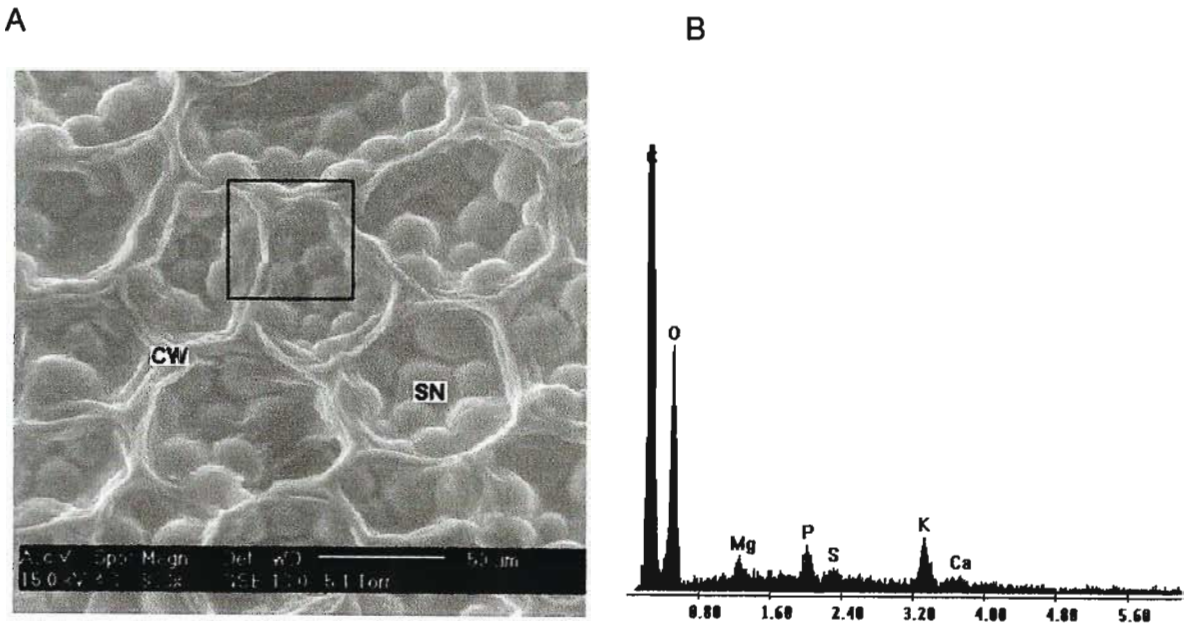
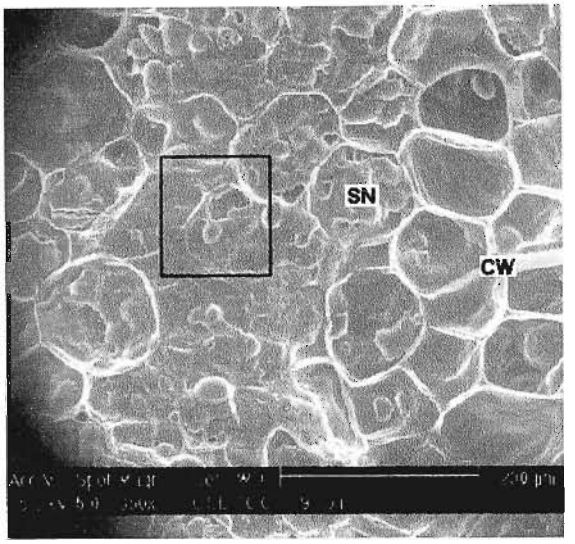


Figure 2.1. Electron micrograph (A) and EDAX spectrum (B) from EDAX analysis of *Phaseolus vulgaris* L. cv. Tongaat dry seed. CW = cell wall; SN = seed nutrient storage organs.

A



B

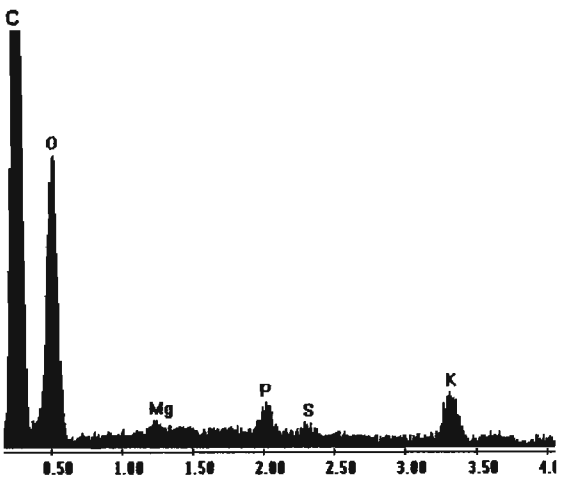


Figure 2.2. Electron micrograph (A) and EDAX spectrum (B) from *Phaseolus vulgaris* L. cv. Tokai dry seed. CW= cell wall; SN= seed nutrient storage organs.

All salt treatments enhanced cotyledonal calcium content (Figure 2.3). Generally there was more nutrient uptake when the seeds were primed than when coated. However there was no significant variation in the efficiency of calcium uptake (ΔCa) among the various treatment structures within each cultivar (Appendix 2). Calcium uptake did however differ significantly between the cultivars (P = 0.023; LSD = 0.1654), with ΔCa of Tongaat being significantly higher – at 0.544% – than that of Imbali and Tokai, which were 0.38% and 0.264% respectively. Since there was no significant difference in calcium uptake between the salts or their concentration, 100mM $Ca(NO_3)_2$ was chosen as the source of Ca^{++} ions in downstream experiments.

The general response of the three cultivars to priming is illustrated in Figure 2.4 where seeds were primed with 100mM $Ca(NO_3)_2$. Differences in seed size may account for the difference in nutrient uptake observed in Figure 2.4. The larger seeded Tongaat seeds (see Table 2.1) absorbed significantly more nutrient than the smaller Tokai and Imbali.

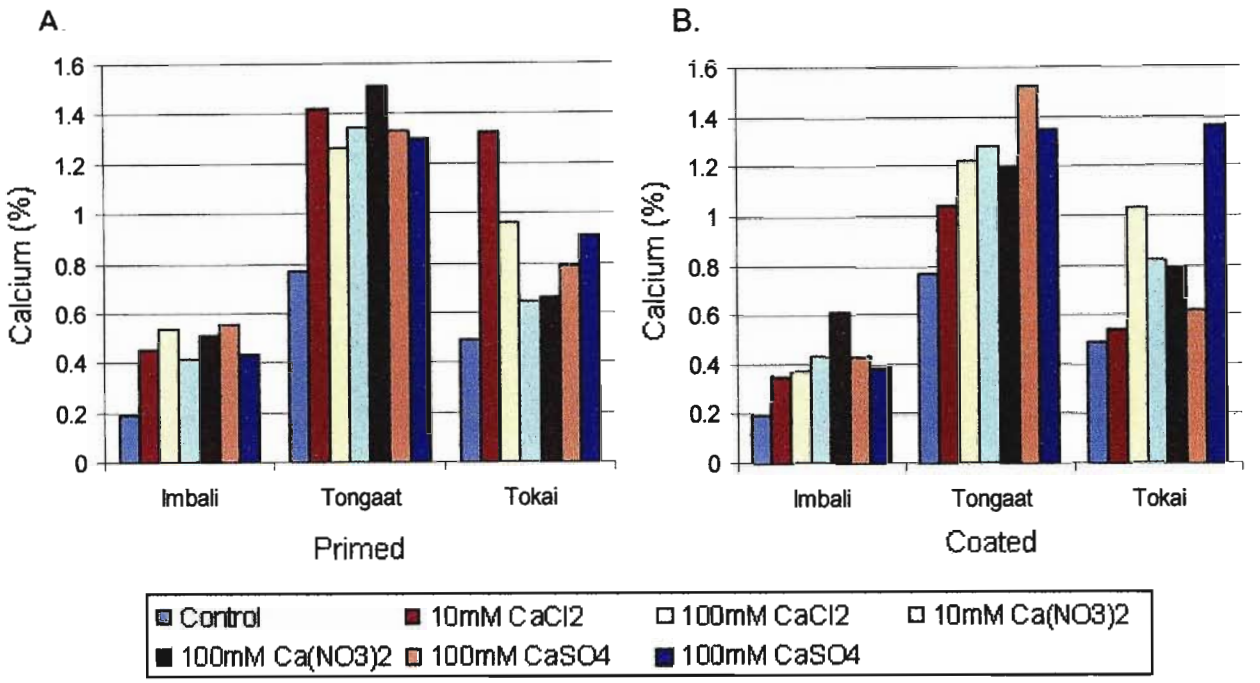


Figure 2.3. Comparison of efficacy of priming (A) and coating (B) enhancement techniques with respect to calcium uptake by cotyledons of *Phaseolus vulgaris* L. cultivars.

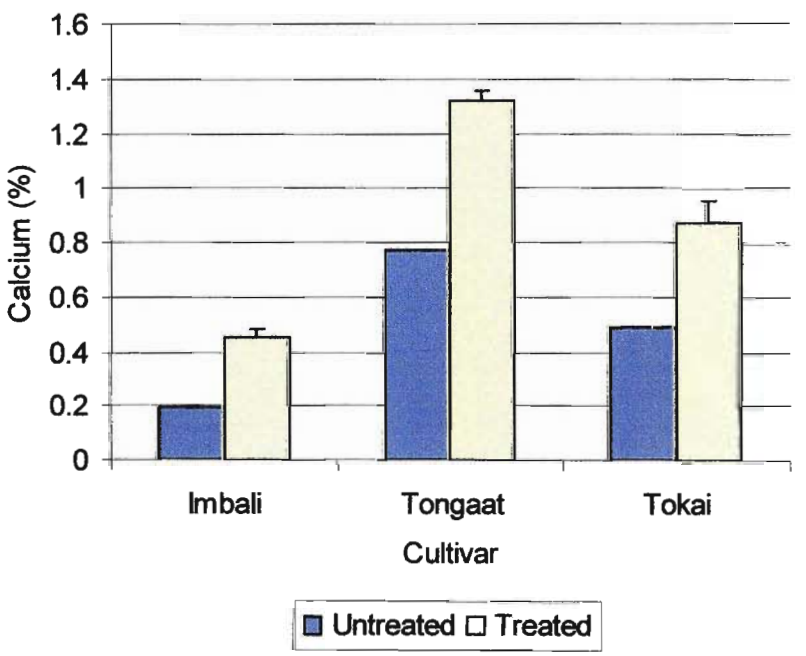


Figure 2.4. Efficacy of calcium uptake when bean seeds were primed with 100mM $Ca(NO_3)_2$. Bars indicate standard error.

2.3.2 Seed quality and vigour

The enhancement methods employed significantly reduced both the germination potential and normal seedling stand ($P < 0.001$; Appendix 3). As Tables 2.3 and 2.4 illustrate, priming had the most detrimental effect on bean vigour.

Table 2.3. Effect of seed mineral enhancement method on germination potential[3]. Seeds were treated with 100mM $Ca(NO_3)_2$. (LSD = 15.12; $P<0.001$)

	Treatment		
Cultivar	Untreated	Coated	Primed
Imbali	90	65.4	53.3
Tongaat	100	90.1	46.7
Tokai	100	80.6	41.7

Table 2.4. Percentage abnormal seedlings after treatment with 100mM $Ca(NO_3)$. (LSD = 15.79; $P< 0.001$)

	Treatment		
Cultivar	Untreated	Coated	Primed
Imbali	0.85	5.6	51.1
Tongaat	0	35.9	60.1
Tokai	0.05	21.7	71.8

Deformities were observed in the emergent seedlings after both priming and coating that would be expected to affect plant emergence and establishment in the natural environment. These included negative geotropism, severe fungal infection, lack of hypocotyl, and absence of a primary root.

[3] Percentage germinated seeds after five days incubation at 25°C.

2.3.3 Effect of calcium treatment on TVC incidence and yield

Various workers have documented evidence that calcium salts lower the incidence of TVC (Dickson *et al.*, 1973; Aqil and Boe, 1977; Dickson and Boettger, 1977; Mazibuko, 2003). In Figure 2.5 this observation is duplicated: the more calcium detected in cotyledon tissue of seeds post-treatment, the less the incidence of TVC. However this could not be qualified by statistical analysis as at 95% confidence level no significant negative correlation was observed (Appendix 4). Similarly, although an inverse relationship between amount of calcium and plant yield could be

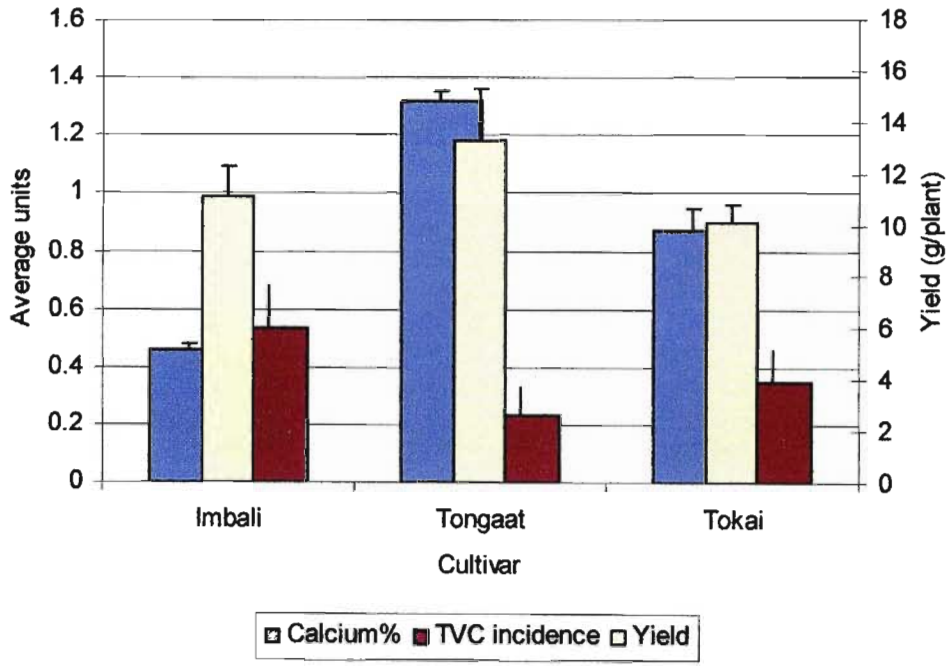


Figure 2.5. Comparison of TVC incidence and plant yield of crops with different cotyledonal calcium content. Seeds were primed with $Ca(NO_3)_2$. TVC incidence expressed as number of cracks per plant. Note: average units = relative Ca (%) or TVC incidence.

observed, statistically the relationship was not significant. There was, however, a significant (r = -0.74) negative correlation between TVC incidence and plant yield in the more severely afflicted Imbali cultivar (Appendix 6),

2.4 Discussion and conclusion

The two most commonly used seed enhancement techniques are coating and priming. The latter is often favoured because the nutrient is absorbed directly into the seed, whereas with coating the nutrient is absorbed into the plant at the onset of germination, at which stage much of the nutrient-containing polymer may have been washed off. However priming also has the major disadvantage of allowing essential storage nutrients to leak out of the seed, thus rendering the technique counter-productive.

In applications where priming is the enhancement technique used, seed size and moisture content have been identified as factors (among others) that affect seed imbibition rates. In her study, Mazibuko (2003) noted that larger seeds imbibed water more rapidly than smaller seeds. This may be due to the larger surface area in contact with the surrounding liquid than smaller seeded varieties.

Initial seed moisture content has also been implicated in water imbibition rates, with the more hydrated seeds having higher water uptake. This observation may be attributed to more solubilization of water soluble storage nutrients which results in more solute concentration – a condition which in turn lowers the water potential within the seed thus allowing for increased water uptake (Hsu *et al.*, 1983).

The results obtained in this trial are in agreement with the above-mentioned observations. Varieties Tongaat and Tokai, being large and containing more moisture (Table 2.1), absorbed more of the calcium-containing solution than Imbali and hence retained more calcium ions. No significant difference was observed between the different calcium salts and salt application within each cultivar.

A second feature observed in these findings was the adverse effect that salt treatment had on seed vigour. As already noted, seed priming often results in leakage of stored nutrients which are essential for plant development. In this study,

there was a general deterioration in seed vigour (as shown by lowered germination potential and high abnormality in seedlings) after priming (Tables 2.3 and 2.4). Seed coating also lowered seedling vigour, though to a lesser degree than priming. The loss in vigour may be due to a loss of trace quantities of vital seed constituents (such as extant mRNAs) needed for germination. The high solute concentration in the coating polymer could create a high water potential within the seed, thus leading to leakage as water moves down the diffusion gradient to the outside.

The lowering of yield in TVC-affected plants is well documented (Aqil and Boe, 1977; Eisinger and Bradford, 1986). This observation is often attributed to, among others, the limited translocation of nutrients from the cotyledons to the nascent seedling, thus compromising seedling establishment. However, with the exception of Imbali, no significant correlation could be found between TVC incidence and lowered plant yield. This maybe attributed to the unreliable method of detection (visual scoring) employed. Similarly, the results of the present study could not affirm that seed calcium content lowered the incidence of TVC – an observation made elsewhere (Dickson *et al.*, 1973; Aqil and Boe, 1977; Dickson and Boettger, 1977; Mazibuko, 2003).

In summary, the larger seeded Tongaat variety with elevated moisture content will more readily absorb nutrient during enhancement exercises. However both the priming and coating techniques lower the germination potential and seedling viability, with priming being the most detrimental. The seeds that do survive the enhancement process, however, have a reduced TVC incidence and higher yield at harvest.

References

Aqil BA, Boe AA (1977) Occurrence of cotyledonal cracking in snap beans and its relation to nutritional status in the seed. HortScience **10**: 509-510

Association of Official Seed Analysts (1992) Seedling evaluation handbook. AOSA Publishers. AOSA, Lincoln, NE

Copeland LO, McDonald MB (1995) Principles of seed science and technology, Ed 3. Chapman and Hall, pp 258-276

Dickson MH, Duczmal K, Shannon S (1973). Imbibition rate and seed composition as factors affecting transverse cotyledon cracking in bean (*Phaseolus vulgaris* L.) seed. Journal of the American Society for Horticultural Science **98**: 509-513

Dickson MH, Boettger MA (1977) Inheritance of resistance to mechanical damage and transverse cotyledon cracking in snap beans (*Phaseolus vulgaris* L.). Journal of the American Society for Horticultural Science **102**: 498-501

Eisinger BA, Bradford, KJ (1986) Role of seed mineral content in the occurrence of cotyledon cracking of snap bean. Journal of the American Society for Horticultural Science **111**: 110-114

Fry CF (1988) The Growing Plant Cell: Chemical and Metabolic Analysis. Longman Scientific & Technical, Essex

Hsu KH, Kim CJ, Wilson LA (1988) Factors affecting water uptake by soybean during soaking. Cereal Chemistry **60**: 208-211

Marschner H (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, New York, pp 285-299

Mazibuko TG (2003) Alleviation of cotyledonal cracking in green beans (*Phaseolus vulgaris* L.) by calcium seed treatment. MSc Thesis. University of Natal, Pietermaritzburg, South Africa

Mazibuko TG and Modi AT (2005) Comparison of osmopriming and seed coating with calcium salts for green bean performance under field conditions. I. Cotyledonal cracking. South African Journal of Plant and Soil **22**: 9-15

Modi AT, Kjonstad TR and White BJ (2004) Comparison of EDX and AA to determine distribution of Ca in green bean (*Phaseolus vulgaris* L.) seed parts following osmopriming. South African Journal of Botany **70**: 298-302

CHAPTER 3

INVESTIGATING THE ROLE OF POLYGALACTURONASE AND PECTIN METHYLESTERASE IN TVC

3.1 Introduction

The cracking of cotyledons in green bean (TVC) is believed to be a result of weakened cell wall strength (Mazibuko and Modi, 2005). The phenomenon has been observed to be accompanied by degradation of the middle lamella and loss of cell adhesion. In most plants pectic polysaccharides are the major constituent of the middle lamella (Cosgrove, 1997) and thus the degradation of pectin polymers is one of the most notable changes of plant tissues with weakened cell walls. To assess whether TVC has an enzymatic origin (as opposed to environmental factors) it is therefore desirable to look at the enzymes responsible for pectin degradation. Moreover, previous examinations have revealed that TVC is an inherent trait and is inhibited by calcium salts (Dickson *et al.*, 1973; Aqil and Boe, 1977; Dickson and Boettger, 1977; Mazibuko and Modi, 2005). An investigation into calcium-inhibited endogenous enzymes that degrade pectin should therefore provide an insight into the role of pectinolytic enzymes in the cracking of cotyledons.

For reasons given previously (Chapter 1) polygalacturonase (PG, EC 3.2.1.15) is the wall-degrading enzyme targeted by the present study. The hypothesis is that PGs from TVC resistant cultivars have a lower activity than those of less resistant ones. The enzyme loosens cell walls by catalysing the hydrolytic cleavage of α-(1,4)-galacturonan linkages in the homogalacturonan component of pectin, releasing GalU residues (van Santen *et al.*, 1999). The released polyuronides (GalU residues) have been observed to be of differing molecular mass depending on the stage of maturity (Wakabayashi *et al.* 2003). The PG-hydrolysed polyuronides in pre-ripe fruit were observed to be of higher molecular mass than those of ripe fruit, indicating that the lower molecular mass of fully ripe avocado fruit required the participation of other

enzymes. It has also been observed that highly methylesterified galacturonan impedes the action of PG (Tans-Kersten *et al.*, 1998). These observations suggest that α-(1,4)-galacturonan hydrolysis is strongly regulated by the level of methylation of the galacturonic acid polymer.

While the main target enzyme in this study is polygalacturonase, the necessity of investigating the role of pectin methylesterase (PME) in TVC becomes apparent when considering the role methylation plays in maintaining cell wall structural integrity. PME [EC 3.1.1.11] catalyses the hydrolysis of ester bonds between the methyl and the C6 carboxyl group of galacturonosyl residues in the galacturonan chain of pectin (Boccara and Chatain; 1989). Methanol is released thus exposing polygalacturonic acid to the action of polygalacturonase. Thus both the degree of esterification of pectin and the activity of PME would therefore determine the activity of PG, whose activity in turn determines the integrity of plant tissue. In view of the abovementioned points, the present study aims to evaluate the role played by pectin methylation, PG, and PME activity in the cracking of bean cotyledons.

3.2 Materials and methods

3.2.1 Plant material and TVC scoring under controlled environment

Seeds from three *Phaseolus vulgaris* L. cultivars (Imbali, Tongaat, and Tokai) were obtained from the School of Agricultural Sciences and Agribusiness (Crop Science Department), University of KwaZulu-Natal, Pietermaritzburg. The seeds were grown in an agricultural growth tunnel in white sand medium supplemented with Hoagland's Nutrient (Appendix 8). At five days after emergence the cotyledons of the seedlings were scored visually for TVC, and the incidence was scored as the average number of cracks per plant. Cotyledons from *P. vulgaris* at dormancy (dry seed prior to germination), VC (cotyledonal stage), R4 (early seed filling), and R6 (late seed filling and physiological maturity) stages of development were examined for enzyme activity and expression.

3.2.2 Extraction cellular material

Seeds from the three green bean cultivars were used for laboratory analysis. The seed coat plus embryonic axis were removed with a scalpel and the remaining cotyledons were surface-sterilized in three washes of 0.05% sodium hypochlorite then rinsed in distilled water. The cotyledons were then ground in liquid nitrogen to a fine powder in a pestle and mortar and transferred to the appropriate extraction buffer (as determined by downstream applications). A modification of the Bradford (Bradford, 1976) protein assay was used to determine the concentration of the protein extracts. Samples (2µl) were added to the protein dye reagent (Bio-Rad® protein assay kit) as per instruction by manufacturer. The mixture was incubated at 60°C for 30min and, after cooling to room temperature, absorbance readings taken at 562nm. A protein standard was prepared similarly and from the linear plot obtained the concentrations of the experimental protein samples were estimated.

3.2.3 Enzyme extraction and determination of activity

3.2.3.1 Polygalacturonase Crude Extract

The lysis buffer contained 9.5M urea, 2% (w/v) CHAPS, 0.8% pharmalyte and 5mM PMSF. For storage purposes the lysis buffer was prepared without the PMSF and, after filter sterilization, kept at -70°C. PMSF was added immediately prior to extraction. To extract protein, the lysis buffer was added to the ground cotyledonal tissue at 2ml: 1g ratio. After shaking for 2hrs at room temperature, the mixture was centrifuged at 32000*g* for 30min at 4°C. The supernatant was retained and stored at -20°C.

3.2.3.2 PG Activity

The activity of polygalacturonase was assayed according to the 2-cynoacetamide method described by Gross (1982). To prepare the substrate 0.1% (w/v) polygalacturonic acid (washed in 80% ethanol prior to use) was dissolved in 0.1M sodium acetate and the pH adjusted to 5.3 with dilute NaOH. An aliquot (25µl) of the

enzyme extract containing 3.75µg of protein was added to 200µl of substrate. The mixture was incubated at 30°C and, at fixed time intervals, the reaction was terminated with 1ml of cold 100mM borate (pH9.0) buffer. To quantify the released reducing groups, 0.2ml of 1% 2-cyanoacetamide was added and the mixture was boiled for 10min. After equilibrating to 25°C, the solution was transferred to a quartz cuvette and the absorbance read at 276nm. Galacturonic acid standards were prepared similarly.

3.2.3.3 PME Crude Extract

Cotyledonal tissue ground in liquid nitrogen was homogenised in cold (4°C) 8.8% (w/v) NaCl pH7.5 and centrifuged at 20000g for 10min. The supernatant was retained and kept at -20°C until used.

3.2.3.4 PME Activity

The activity of pectin methylesterase was assayed by adding 20µl enzyme extract (containing 50µg protein) to 500µl of substrate [0.5% citrus pectin in 8.8% NaCl, pH7.5]. 50µl bromothymol blue (pH7.5) was used as the chemical indicator for the change in pH brought about by the release of carboxyl groups. The change in colour of the reaction mixture was measured spectophotometrically at 620 nm over a period of 160 sec. PME activity was shown as a decrease in absorbance reading over time and were scored as coefficient of determination (r^2).

3.2.4 Pectin extraction

Pectin extraction was performed according the guidelines set by Dinu (2001). Ethanol insoluble material (EIM) was obtained by washing ground cotyledon powder in 80% ethanol, and the ethanol was removed in a vacuum oven at 80°C. To extract pectin from the polysaccharides the EIM was stirred in Millipore water at 25°C for 1 hour and centrifuged at 3000g at room temperature for 20 minutes. The residue was re-suspended in 1% ammonium oxalate and stirred for 1 hr at 25°C, and centrifuged

at 3000g at room temperature for 20 min. The pectin-containing supernatant was retained.

3.2.5 Determination of the degree of methylation of pectin

The procedure described by Klavons and Bennett (1986) was used to determine the degree of methylation (DM) in the extracted pectin. 25ml of 1.0 N potassium hydroxide was added to 25ml aliquots of pectin extracts containing 50µg of protein. After incubation at room temperature the solutions were neutralised with dilute phosphoric acid to pH7.5. Solutions containing 1ml potassium phosphate buffer (0.1M, ph7.5) and 50µg pectin extract from the different cultivars were placed in Pyrex screw-cap culture tubes and 1ml aliquots of crude PME extracts from the R4 growth phase[4] were placed into each tube. After incubation at room temperature for 15min, a 2ml solution containing 0.02M 2,4-pentanedione in 2M ammonium acetate and 0.05M acetic acid was added to the tubes and the mixture was vortexed briefly to mix the contents. The tubes were then placed in a water bath at 60°C for 15min and cooled to room temperature. Methanol standards in the range of 1-20 µg/ml were prepared similarly with the exclusion of pectin. Aliquots of the solutions were transferred into quartz cuvettes and their absorbencies were measured at 412nm against a blank containing 0.1M phosphate buffer and the PME extract.

3.2.6 Statistical Analysis

The activity of PG was defined as the amount of galacturonic acid released in the reaction vial over time. To test whether the increase is statistically significant (whether there is real activity as opposed to chance changes in the spectrophotometer readings), the correlation analysis tool of MS Word was used (p = 0.05). Similarly, the change in pH in the PME activity reaction (as shown by a decrease in absorbance readings) was subjected to the same test.

---

[4] PME extracts showed the most activity at this stage. See Results section below.

3.3 Results and discussion

3.3.1 Pectin Methylation

Cotyledons were collected five days after emergence and their TVC was scored before laboratory analysis. The TVC incidence was then contrasted with the degree of methylation of the cotyledonary cell walls (Figure 3.1).

There was an inverse relationship between TVC incidence and DM in all the cultivars. Pectin from the more TVC afflicted Imbali cv. was least esterified and that of Tongaat showed the greatest degree of methyl esterification. This finding is in agreement with observations made by other workers that the least esterified cell walls are more vulnerable to enzymatic cleavage.

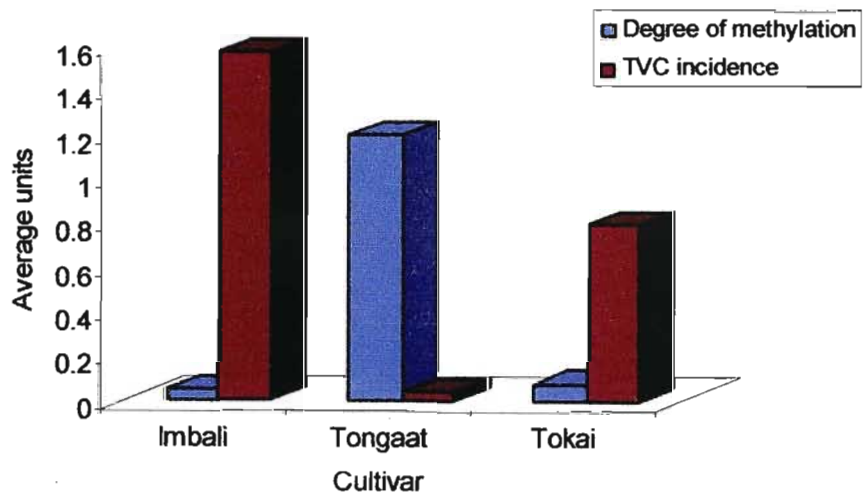


Figure 3.1. Comparison of degree of methylation and TVC incidence. Degree of methylation given as µg/ml methanol/µg pectin, and TVC incidence scored as number of cracks per plant.

It was expected that PME activity would follow a similar trend in the analysis, i.e., that the least methylated cultivar would have the most active PME. The present results did not support this theory, as shown by the lack of any observable relationship between enzyme activity and degree of methylation (Figure 3.2). The lack of correlation between PME activity and release of methanol from the pectin could be due to the fact that PME activity was measured using commercial pectin whereas in the estimation of DM methanol was liberated from crude preparations of pectin. The PME of each variety is possibly better suited to enzymatic cleaving of its own native pectin than that produced by other organisms.

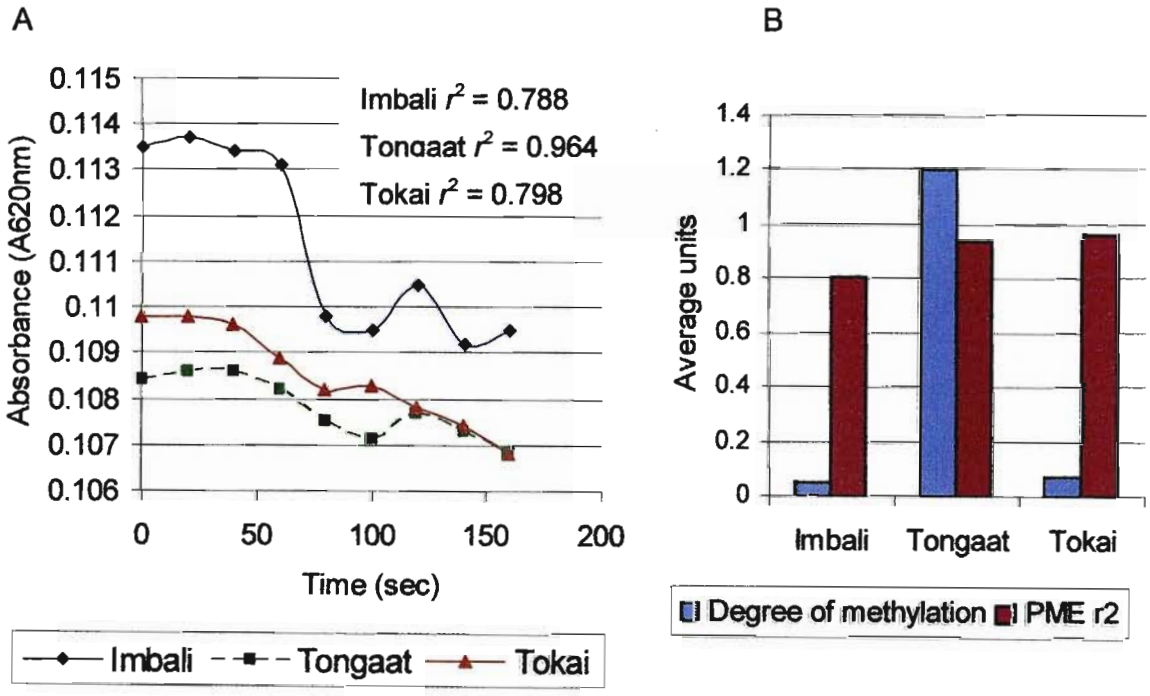


Figure 3.2. Comparison between pectinmethylesterase (PME) activity and degree of methylation (DM). (A) is PME reaction kinetics and (B) shows the comparison between the rate of enzyme activity and DM. Coefficients of determination indicate the strength of the correlation between change in absorbance and progress of time, and thus the rate of activity. Degree of methylation given as µg/ml methanol/µg pectin.

3.3.2 Polygalacturonase Activity

Figures 3.2 and 3.3 show PG activity in the various growth stages examined. PG activity was detected in all the cultivars at the VC stage. Although it is the stage where most cases of TVC become apparent, increased enzyme activity at the VC stage is expected and probably has no bearing on the propensity of the cotyledons to crack. At this stage the phanerocotylar cotyledon actively mobilizes the storage nutrients to the nascent seedling and therefore the activity of pectin hydrolysing enzymes is high. Further evidence in support of this assertion is the presence of TVC in cotyledons isolated from quiescent seeds as is displayed upon water imbibition (Mazibuko, 2003). Therefore the underlying causes of cracking are already present in the seed/cotyledons prior to germination.

In the stages where wall motif predetermines the occurrence of TVC after water imbibition – the R4, R6 and dormancy stages where the seeds (and thus cotyledons) are developed and matured – PG activity was observed to be coincident with the susceptibility level of each cultivar. The more susceptible Imbali cultivar retained high activities of PG at all the stages examined. The PG of the most resistant Tongaat cultivar was only detected in the VC stage of development.

The presence of PG activity at the R4 stage of development in the intermediate Tokai cultivar (which is lacking in the resistant Tongaat), and the corresponding loss of resistance to TVC (as shown by a higher TVC incidence), suggests that R4 is the crucial stage that determines the plant's propensity to TVC. This study proposes that it is the protracted activity of PG into the seed development and maturity stages that lay the foundations for TVC. The continued removal of galacturonic acid residues in the pectin structure at these stages may compromise anabolic processes thereby rendering the cell walls weak.

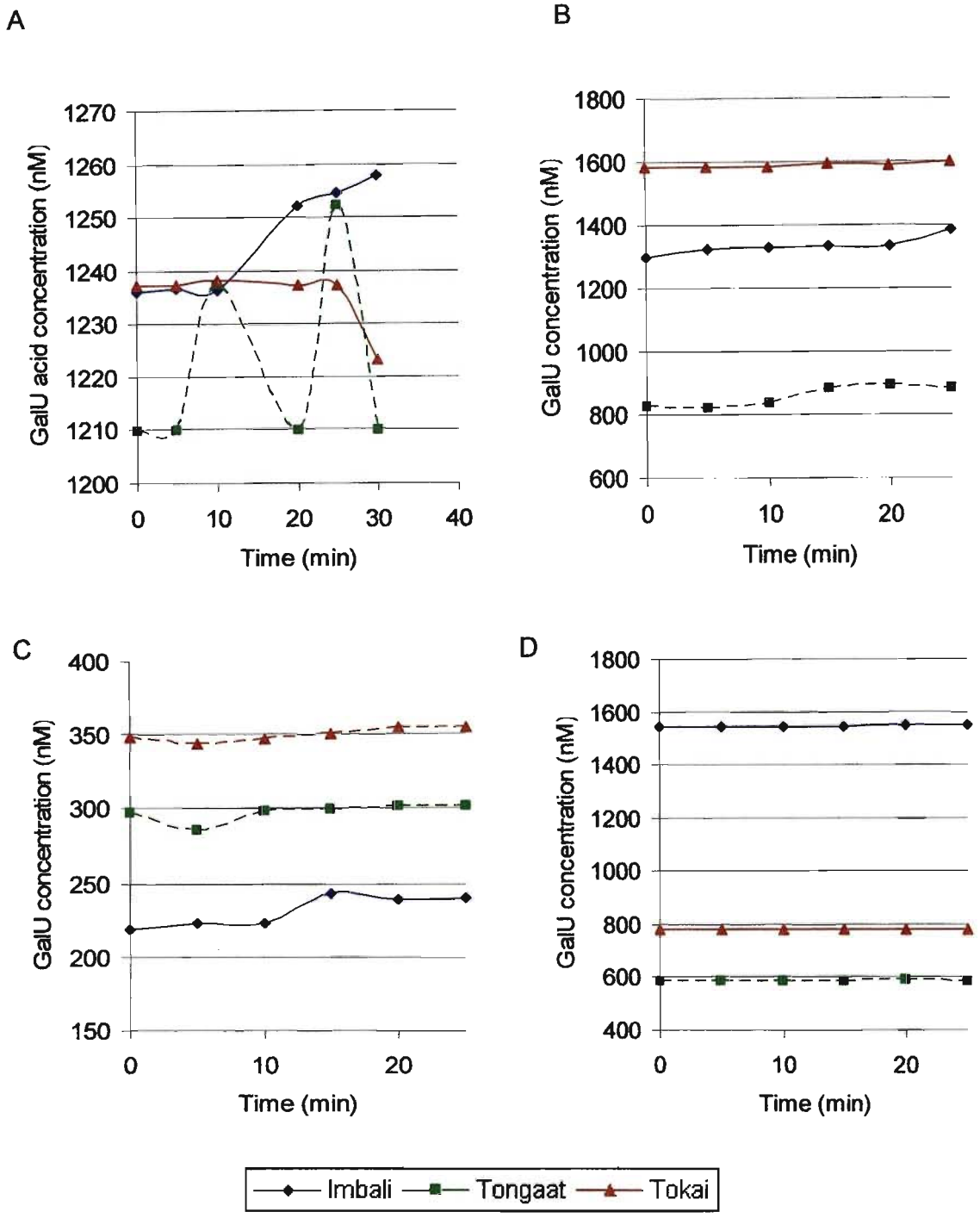


Figure 3.3. Rate of activity of cotyledonary polygalacturonase (PG) extracted from (A), dormant; (B), VC; (C), R4; and (D), R6 stages of growth. The rate of activity is defined as the nM galacturonic acid residues released from 1% polygalacturonate per minute.

Table 3.1. Coefficients of determination (r^2) for polygalacturonase activity in cotyledons at different stages of growth.

	Stage of growth			
Cultivar	Dormancy	VC	R4	R6
Imbali	0.92	0.801	0.765	0.853
Tongaat	0.68	0.795	0.341	0.185
Tokai	0.391	0.768	0.695	0.545

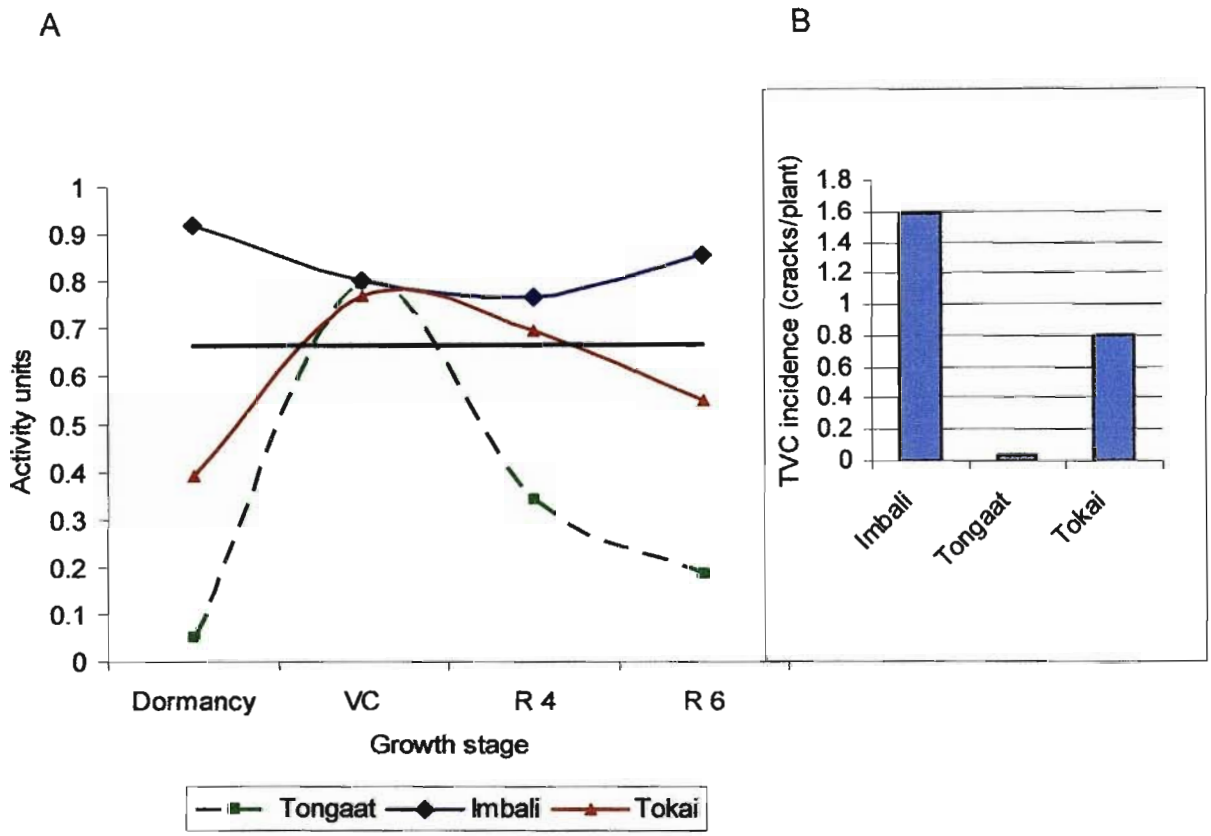


Figure 3.4. Changes in cotyledonal polygalacturonase activity during growth cycle of *Phaseolus vulgaris* cvs (A) contrasted with TVC incidence (B). Activity units are r^2 values of nM galacturonic acid residues released from 1% polygalacturonate per minute. The horizontal black line demarcates the limits of $r^2_{critical}$ above which activity is statistically significant.

3.3.3 Pectin Methylesterase Activity

In contrast to the PG activity curve, no trend was observed between TVC incidence and PME activity in the TVC crucial stages of growth (Fig. 3.5 and Appendix 10). PME activity rose exponentially and reached a peak at the VC and R4 stages then dropped off. The PME does not catalyse the hydrolysis of pectin directly but prepares the pectin for PG digestion by removing methyl groups from the highly methylesterified pectin polymers. Pectin methylation is necessary in wall build-up as the polysaccharides have to be transported in an insoluble form to the site of wall building. High activity of PME at the R4 stage would therefore result in de-esterification which would ultimately result in weaker cell walls thus contributing to TVC. This study however found no relationship between PME activity and TVC incidence in the stages of development.

Table 3.2. Coefficients of determination (r^2) for pectin methylesterase activity in cotyledons at different stages of growth.

		Stage of growth		
Cultivar	Dormancy	VC	R4	R6
Imbali	0.301	0.788	0.811	0.646
Tongaat	0.006	0.798	0.970	0.844
Tokai	0	0.96	0.957	0.644

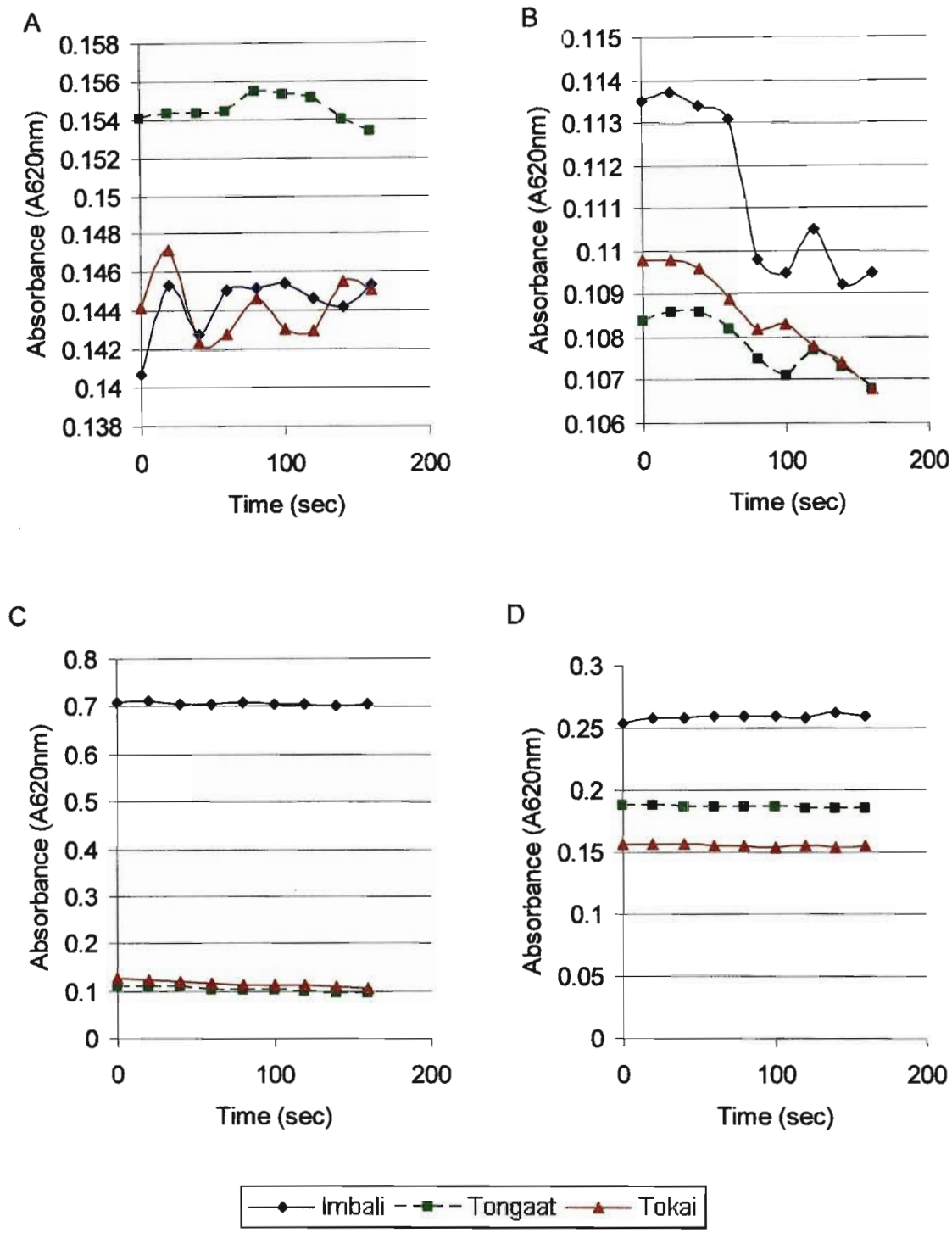


Figure 3.5. Rate of activity of pectin methylesterase (PME) extracted from (A), dormant; (B), VC; (C), R4 and (D), R6 stages of growth. The rate of activity is defined as the r^2 change in pH at A620nm.

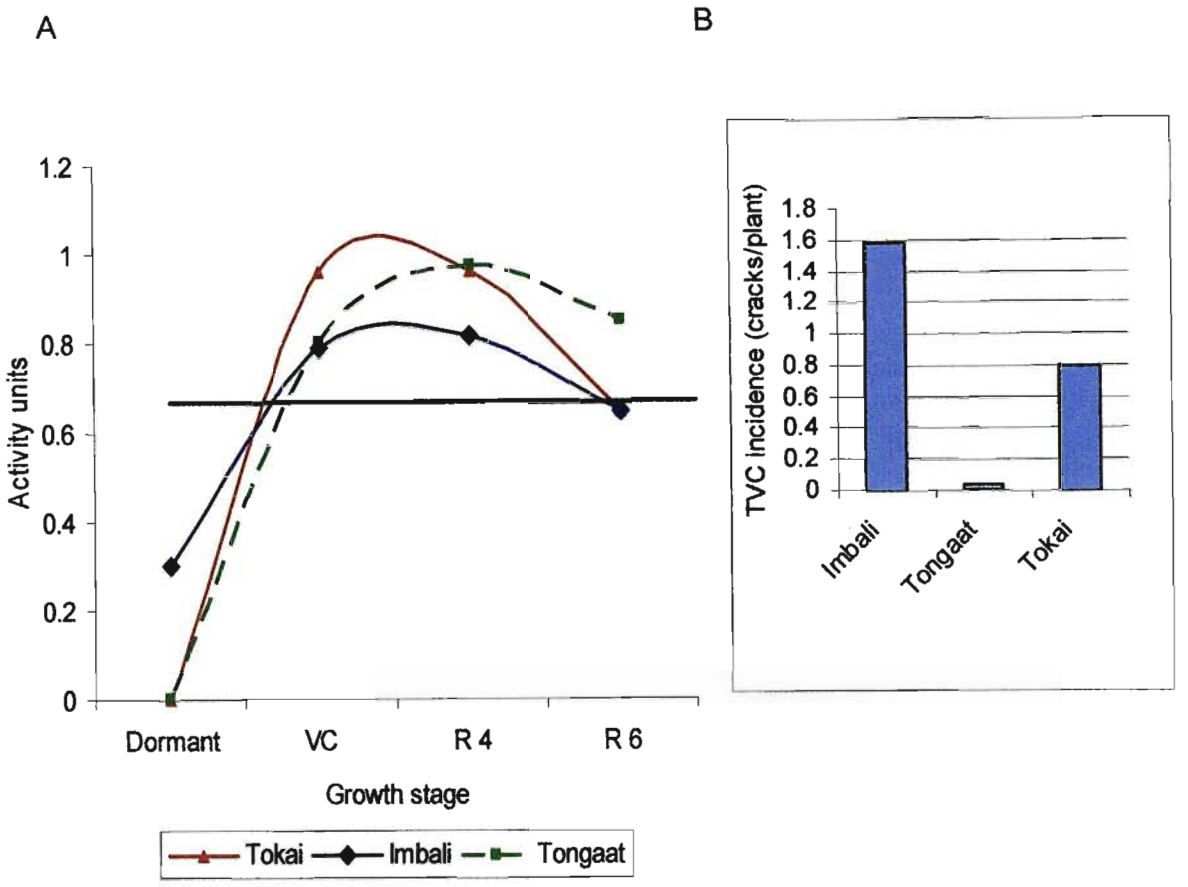


Figure 3.6. Changes in cotyledonal pectin methylesterase during growth cycle of *Phaseolus vulgaris* cvs (A) contrasted with TVC incidence (B). PME activity defined as r^2 change in pH at A620nm. The horizontal black line demarcates the limits of $r^2_{critical}$ above which activity is statistically significant.

3.4 Conclusion

Polygalacturonase (PG) and pectin methylesterase (PME) are known to act in tandem in the degradation of methylesterified pectin. The methyl groups on the galacturonic polymer serve to protect pectin from degradation by making cleavage sites unavailable to pectinolytic enzymes such as PG. PME facilitates the hydrolysis of the galacturonic acid component of pectin by removing methyl groups, thus exposing the cleavage site.

Tissue with structurally weak cell walls, such as that of TVC afflicted cotyledons, is expected to have higher PG and PME activity, and/or have a lower degree of methylation than structurally intact tissue. In the cultivars investigated the activity of PME could not be directly linked to level of susceptibility to TVC. On the other hand PG activity in the R4 stage of growth was determined to be crucial to propensity to TVC. PG activity in the least TVC affected cultivar (Tongaat) was only detected in appreciable levels in the VC stage, whereas the most affected Imbali cultivar had high PG activity in all the stages of growth.

In view of the adverse effects of coating or priming seed (at which stage wall motif is probably already set for cracking), an alternative would be to provide the seeds with Ca^{++} ions as they form at R4 stage. However at present this may prove to not be a viable option due to the higher costs involved, and continued breeding of TVC-resistant cultivars may be more desirable.

References

Aqil BA, Boe AA (1977) Occurrence of cotyledonal cracking in snap beans and its relation to nutritional status in the seed. HortScience **10**: 509-510

Boccara M, Chatain V (1989) Regulation and role in pathogenicity of *Erwinia chrysantheni* 3937 pectin methylesterase. Journal of Bacteriology **171**: 4085-4087

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry **72**: 248-254

Cosgrove DJ (1997) Assembly and enlargement of the primary cell wall in plants. Annual Review of Cell Developmental Biology **13**: 171-202

Dickson MH, Duczmal K, Shannon S (1973) Imbibition rate and seed composition as factors affecting transverse cotyledon cracking in bean (*Phaseolus vulgaris* L.) seed. Journal of the American Society for Horticultural Science **98**: 509-513

Dickson MH, Boettger MA (1977) Inheritance of resistance to mechanical damage and transverse cotyledon cracking in snap beans (*Phaseolus vulgaris* L.). Journal of the American Society for Horticultural Science **102**: 498-501

Dinu D (2001) Extraction and characterization of pectins from wheat bran. Romanian Biotechnological Letters **6**: 37-43

Gross KC (1982) A rapid and sensitive method for assaying polygalacturonase using 2-cyanoacetamide. HortScience **17**: 933-934

Klavons, J.A. and R.D. Bennett. (1986). Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. Journal of Agriculture and Food Chemistry **34**: 597-599

Mazibuko TG (2003). Alleviation of cotyledonal cracking in green beans (*Phaseolus vulgaris* L.) by calcium seed treatment. MSc Thesis. University of Natal, Pietermaritzburg, South Africa

Mazibuko TG and Modi AT (2005) Comparison of osmopriming and seed coating with calcium salts for green bean performance under field conditions. I. Cotyledonal cracking. South African Journal of Plant and Soil **22**:9-15.

Tans-Kersten J, Guan Y, Allen C (1998) *Ralstonia solanacearum* pectin methylesterase is required for growth on methylated pectin but not for bacterial wilt virulence. Applied Environmental Microbiology **64**: 4918-4923

van Santen Y, Benen JAE, Schroter KM, Kalk KH, Armand S, Visser J, Dijkstra BW (1999) 1.68 Å crystal structure of endopolygalacturonase II from *Aspergillus niger*

and identification of active site residues by site- directed mutagenesis. The Journal of Biological Chemistry **274**: 30474-30480

Wakabayashi K, Hoson T, Huber DJ (2003) Methyl de-esterification as a major factor regulating the extent of pectin depolymerization during fruit ripening: a comparison of the action of avocado (*Persea americana*) and tomato (*Lycopersicon esculentum*) polygalacturonases. Journal of Plant Physiology **160**: 667-73

CHAPTER 4

MOLECULAR AND BIOCHEMICAL ANALYSIS OF POLYGALACTURONASE AND PECTIN METHYLESTERASE IN GREEN BEAN COTYLEDONS

4.1 Introduction

Polygalacturonase (PG; EC 3.2.1.15) and pectin methylesterase (PME; EC 3.1.1.11) are enzymes known to be involved in the degradation of pectin, the cell wall component into which cellulosic material is embedded. As the 'glue' that holds cellulose microfibrils together, pectin plays an important role in maintaining wall integrity. The activities of the pectinolytic enzymes in plant tissues showing structural disintegration should therefore be investigated if an endogenous cause is suspected, as is the case with cotyledonal cracking (see Chapter 1).

In vivo, PG and PME have synergistic roles in the degradation of pectin. Native pectin is protected from PG degradation by methyl esterification of the galacturonic acid chain resulting in methyl-D-galactopyranosyl residues (Johansson *et al.*, 2002). PME catalyses the de-esterification of pectin by hydrolysing carboxylates and methanol from the methyl-D-galactopyranosyl residues. The de-esterified galacturonic acid residues form cross links via the calcium divalent forming a gel matrix that maintains intercellular adhesion. By removing methyl groups from galacturonic acid chains, PME prepares the pectin substrate for pectinolytic enzymes such as PG which cannot access the cleavage site of methylated pectin polysaccharides.

Pectin degradation by PG has been linked to various developmental processes, and has also been implicated in several conditions that involve tissue degradation. Likewise the abundance of PME in processes requiring wall modification has been observed (Ren and Kermode, 2000; Micheli *et al.*, 2000; Pilatzke-Wunderlich and Nessler, 2001). For reasons given previously, PG hyperactivity is suspected in

transverse cracking (TVC) of bean cotyledons (Chapters 1 and 3). The present study was aimed at using molecular techniques to isolate, and hence, trace the expression levels of the pectinolytic enzymes PG and PME from cotyledons of green bean cultivars. Comparison between enzyme activity and expression level, contrasted with estimated susceptibility to TVC, should lend more weight to the thesis that PG is the causal agent of TVC.

4.2 Materials and methods

4.2.1 Plant Material

Seeds from three *Phaseolus vulgaris* L. cultivars (Imbali, Tongaat, and Tokai) were obtained from the School of Agricultural Sciences and Agribussiness (Crop Science Department), University of KwaZulu-Natal, Pietermaritzburg. The seeds were grown in an agricultural growth tunnel in white sand medium supplemented with Hoagland's Nutrient (calcium nitrate provided the Ca^{++} ions, see Appendix 8). Cotyledons from *P. vulgaris* at dormancy, VC, R4, and R6 stages were used for enzyme analysis. At 10 days after emergence the cotyledons of the seedlings were scored visually for TVC, and from the incidence the cultivars were rated as resistant, moderately resistant, or susceptible.

In order to assess the effect of calcium salts on the seed proteome and PG activity, seeds were treated with calcium nitrate in the method described in Chapter 2. Treatment structure included controls where seeds were untreated (UTD), imbibed with water only (H_2O), and coated with a salt-less polymer (CoatC). Experimentals were seeds coated or primed with $Ca(NO_3)_2$ (where coated= C, and primed= P) at concentrations of 10nM and 100mM.

Thin cross-sections of the cotyledon tissue were collected for the determination of calcium content. The environmental scanning electron microscope (ESEM) (Philips XL30) with the energy dispersive atomic X-ray analysis (EDAX) program was used

to analyze the tissue calcium content. Specimens were analysed at low vacuum mode (1torr) and area scanning was allowed for 100 livetimesecs per reading. The quantitative microanalysis facet of EDAX was used to quantify the calcium content of the scanned area.

4.2.2 Enzyme extraction and determination of activity

Crude extracts of PG and PME were obtained and their activities assayed according to the method described by Gross (1982) for PG activity, and Hagerman and Austin (1986) for PME activity (see also Section 3.2.3). The strength of the correlation between reaction time and amount of product produced (r^2), co-efficient of determination) was used to represent the rate of enzyme activity.

4.2.3 Gel electrophoresis and zymogram staining

Electrophoresis was according to the method described by Laemmli (1970). Protein samples were boiled in 2 x sample buffer for 5min and analyzed by SDS/PAGE in 10% (w/v) gels. At completion of electrophoresis the protein bands were stained with Coomassie Brilliant Blue. To increase the efficiency of refolding into an active enzyme for zymogram staining, sample boiling time was reduced to 1min before loading on to a 10% SDS/PAGE gel, which contained 0.3% (w/v) polygalacturonic acid (Sigma). Denaturing agent SDS was removed by washing gels for 3hrs in 1M NaCl at room temperature. Subsequently the gels were incubated overnight at 30°C in 10mM sodium acetate buffer (pH5.3) for the PG zymogram, and 10mM NaCl (ph7.5) for PME zymogram. Gels were then stained with 0.05% (w/v) Ruthenium Red (Sigma) for 20min and washed with distilled water until lytic bands appeared.

4.3 Results

4.3.1 TVC sensitivity rating and calcium content

Visual analysis of cotyledons of 10 day old seedlings revealed that the Imbali cultivar had the highest TVC incidence, Tongaat the lowest, and Tokai an intermediate incidence. Figure 4.1 illustrates the inverse relationship displayed between calcium content and TVC incidence.

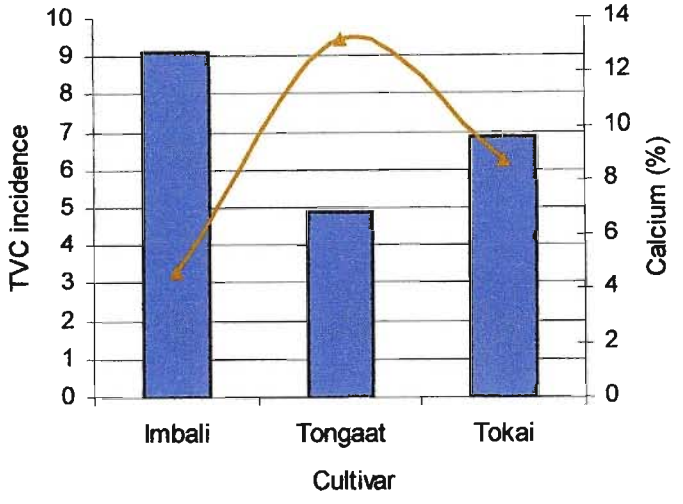


Figure 4.1. Incidence of TVC (bars) in relation to calcium content (line) in seedlings of three *P. vulgaris* cultivars. The incidence was calculated as the average number of cracks per plant.

4.3.2 Zymogram analysis

Zymogram experiments were conducted in order to characterize the active bands in the cotyledonal proteomes of the cultivars. Enzyme activity was confirmed in zymogram gels of both enzymes. The PG zymogram showed lytic bands of about 45kDa while those of PME were approximately 30kDa. No differences in lytic band position of the same enzyme were observed between the cultivars.

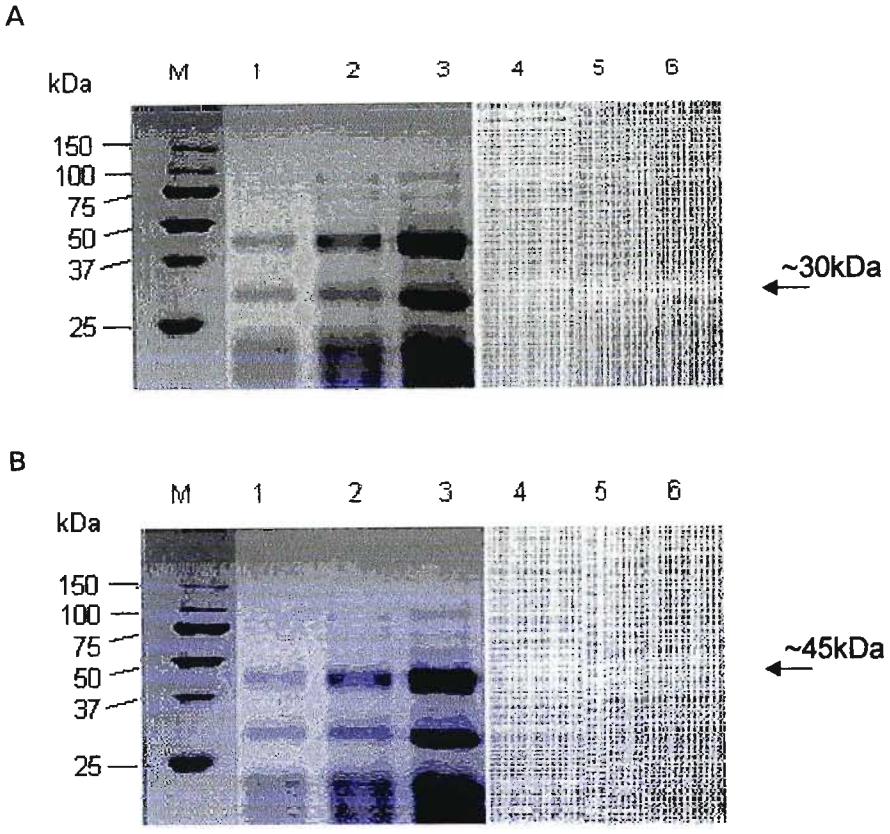


Figure 4.2. One-dimensional SDS gel electrophoresis of the bean cultivars Imbali, Tongaat and Tokai (lanes 1, 2, 3) and their zymogram gels in the same order on the left. The zymogram in A shows the position of PME (indicated by arrow) while B shows that of PG. Each lane contains ~3.75µg of protein.

4.3.3 Effect of calcium on polygalacturonase enzyme activity and expression

Analysis of enzyme activity and expression was done on seeds at dormancy treated with calcium nitrate. Statistics of enzyme activity are presented in Tables 4.1, 4.2 and 4.3, and proteome analysis are shown in the electrophoresis gels in Figures 4.3, 4.4, and 4.5. Activity was detected in the Imbali enzyme extract controls, but was absent where salt was administered (Except at the 10mMP experimental where activity was also detected. This suggests that inactivation of Imbali PG *in vitro* requires priming with $Ca(NO_3)_2$ at levels above 10mM). Activity was absent for Tongaat and Tokai in both controls and experimentals. Thick banding of the 45kDa

band prohibited analysis of the change in expression after seed treatment. A change in banding pattern was however observed in seeds imbibed in water (prominent in Figures 4.3 and 4.5). This change could be a result of water soluble nutrients dissolving in presence of water. The moisture may have triggered the onset of germination and the dissolved nutrients translocated out of the cotyledons to the embryonic embryo. Alternatively, the period of imbibition (2hrs) may have been too long and allowed the dissolved nutrients to leach out of the cotyledons.

Table 4.1. Imbali seed regression statistics of polygalacturonase activity after $Ca(NO_3)_2$ treatment.

	Dry	H_2O	CoatC	10mMC	100mMC	10mMP	100mMP
r	0.711	0.737	0.305	0.159	0.202	0.803	0.661
r^2	0.506	0.543	0.093	0.025	0.040	0.645	0.437
$r_{critical}$	0.666	0.666	0.666	0.666	0.666	0.666	0.666
F significance	0.017	0.025	0.616	0.797	0.744	0.018	0.224

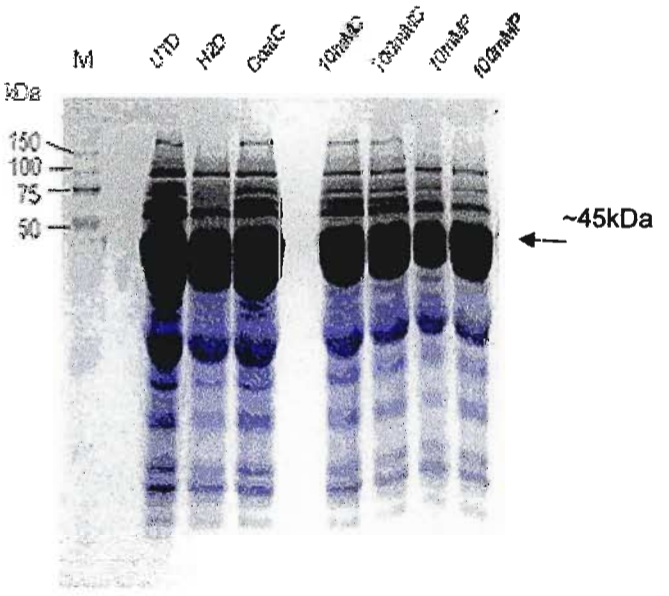


Figure 4.3. Effect of cotyledonal calcium enhancement on Imbali PG expression and activity. Calcium nitrate was used in the treatment. Arrow on right of gel points to PG position.

Table 4.2. Tongaat seed regression statistics of polygalacturonase activity after $Ca(NO_3)_2$ treatment.

	Dry	H_2O	CoatC	10mMC	100mMC	10mMP	100mMP
r	0.354	0.778	0.141	0.275	0.394	0.597	0.305
r^2	0.125	0.606	0.020	0.076	0.155	0.357	0.093
$r_{critical}$	0.878	0.878	0.878	0.878	0.878	0.878	0.878
F significance	0.558	0.120	0.820	0.653	0.511	0.286	0.616

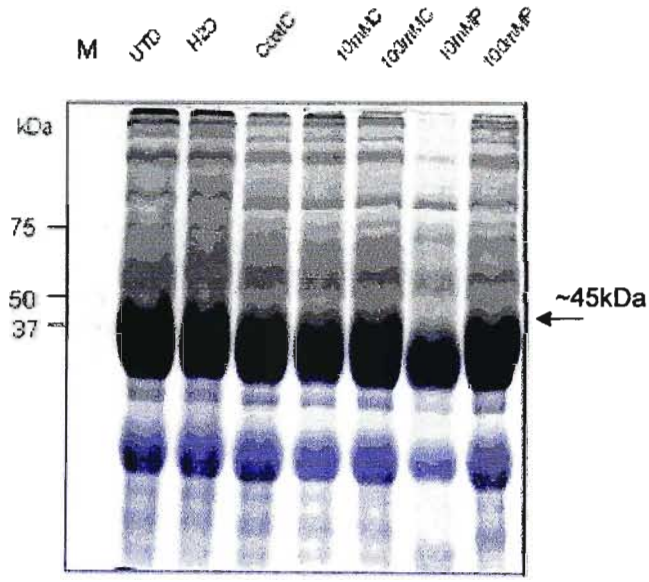


Figure 4.4. Effect of cotyledonal calcium content on Tongaat PG activity and *in vivo* expression. Arrow indicates PG position.

Table 4.3. Tokai seed regression statistics of polygalacturonase activity after $Ca(NO_3)_2$ treatment.

	Dry	H_2O	CoatC	10mMC	100mMC	10mMP	100mMP
r	0.782	0.430	0.498	0.394	0.663	0.538	0.479
r^2	0.611	0.185	0.248	0.155	0.439	0.290	0.897
$r_{critical}$	0.878	0.878	0.878	0.878	0.878	0.878	0.878
F significance	0.117	0.469	0.392	0.511	0.222	0.348	0.142

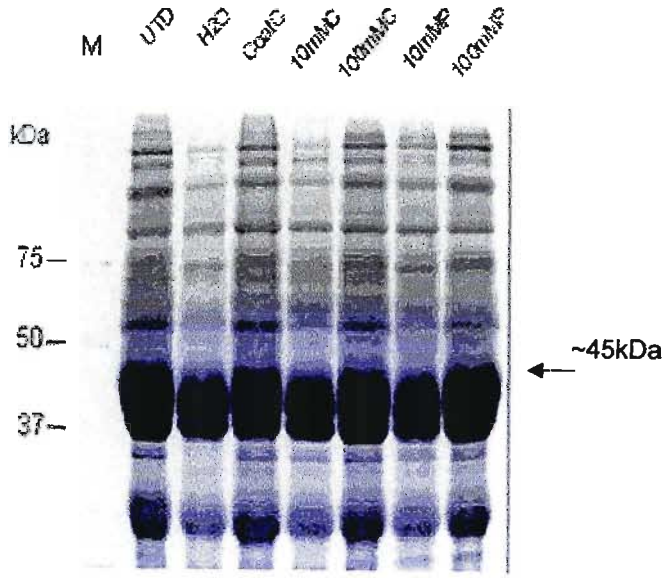


Figure 4.5. Effect of cotyledonal calcium enhancement on Tokai PG expression and activity. Arrow on right of SDS gel indicates position of PG.

4.3.4 Change in activity and expression levels of polygalacturonase and pectin methylesterase through bean growth cycle

As Figures 4.6, 4.7 and 4.8 reveal, PME activity followed a relatively constant metabolism curve in the growth stages[5] investigated. The gradual increase in PME activity in the initial stage of development was replaced by an exponential increase at the VC stage. PME activity reached a peak at the R4 stage then dropped to dormancy levels at R6. The rates of PG activity were at maximum in the Tokai and Tongaat cultivars at VC stage and very little activity was detected at R6 (Figs 4.7 and 4.8). In contrast, PG activity in the Imbali cultivar was at high levels in the D, R4 and R6 stages but dropped at VC (Fig. 4.6).

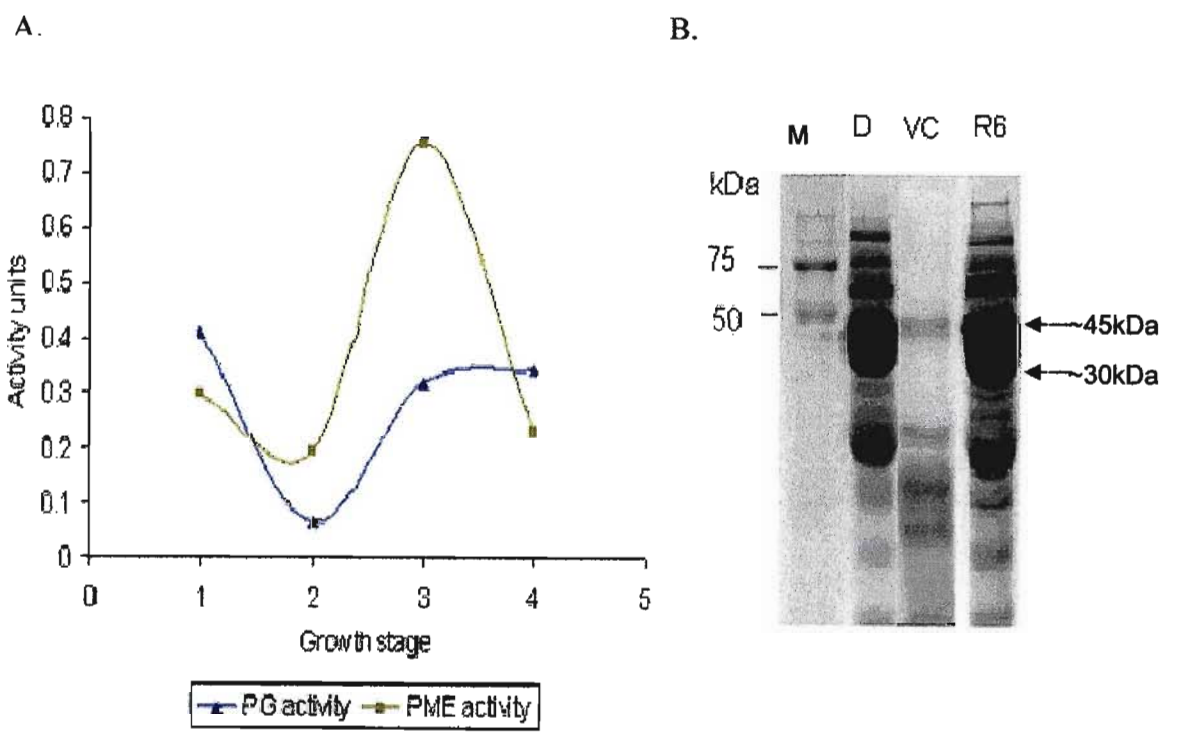


Figure 4.6. Activity (A) and expression (B) of pectin methylesterase (PME) and polygalacturonase (PG) in cotyledon of Imbali bean variety during growth cycle (1 = D, 2 = VC; 3 = R4 ; 4 = R6).

[5] Stages: 1= D (dormancy); 2= VC stage; 3= R4 stage; 4= R6 stage. Protein bands from samples of the R4 stage were too faint to capture photographically and were excluded.

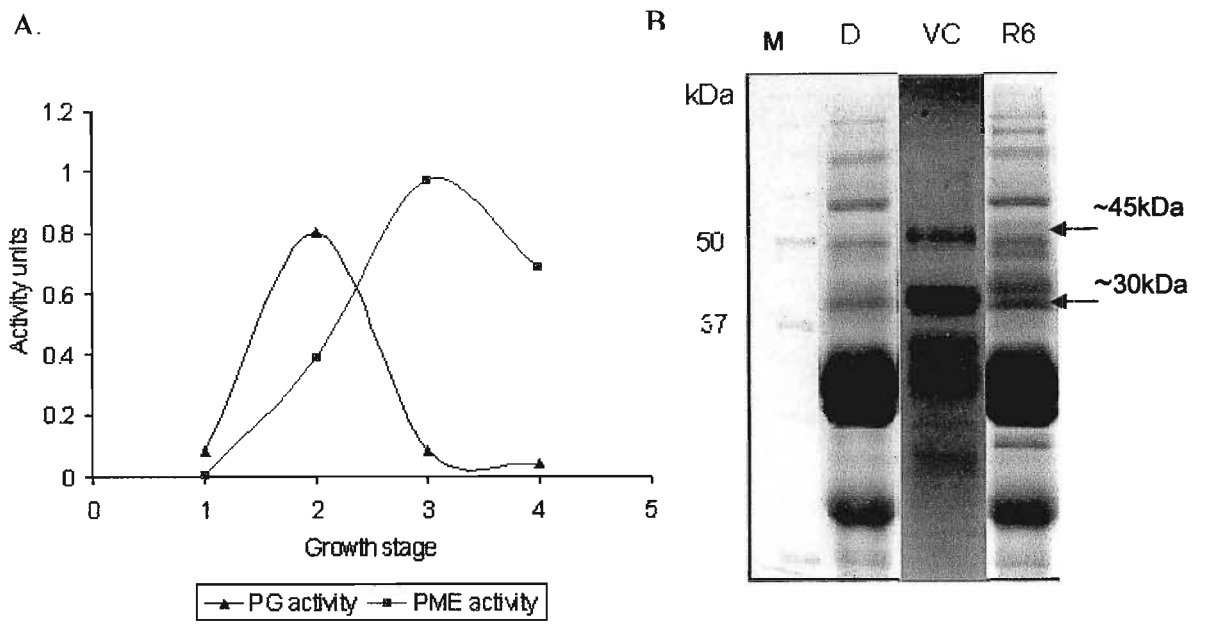


Figure 4.7. Activity (A) and expression (B) of pectin methylesterase (PME) and polygalacturonase (PG) in cotyledon of Tongaat bean variety during growth cycle.

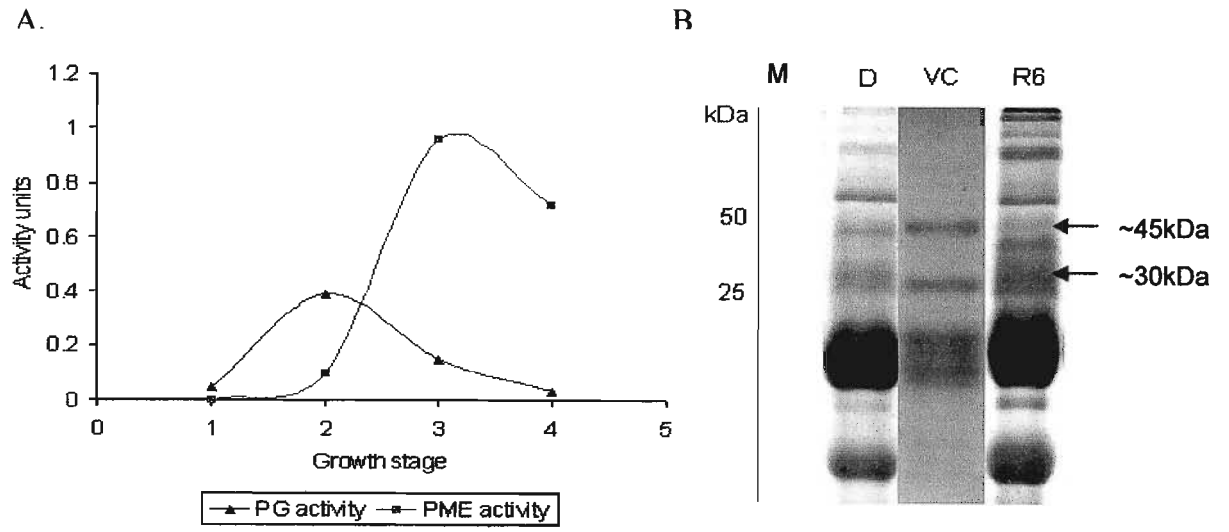


Figure 4.8. Activity (A) and expression (B) of pectin methyl esterase (PME) and polygalacturonase (PG) in cotyledon of Tokai bean variety during growth cycle.

4.4 Discussion and Conclusion

The three cultivars investigated – Imbali, Tongaat and Tokai – were designated susceptible, intermediate and resistant respectively according to their TVC incidence (Figure 4.1). The stages of bean development examined included dormancy and the VC, R4 and R6 where cotyledonal material is available. With respect to TVC, enzyme kinetics are most crucial at dormancy (quiescence), R4 and R6 stages since it is at these stages that structural integrity of the cell walls is essential. At VC the stored nutrients and enzymes are translocated to the rapidly growing seedling, and cotyledonal cracking probably results from pre-existing structural weaknesses. This assertion is supported by the fact that afflicted cotyledons from quiescent seeds exhibit cracks when imbibed with water, as has been observed by other investigators (Mazibuko, 2003).

The change in banding pattern of the more TVC-prone Imbali and Tokai proteomes (Figures 4.3 and 4.5) could be an indication that the cultivars have more water soluble proteins in their cotyledons than the more resistant Tongaat cultivar. Upon water uptake the dissolved nutrients would lower the water potential within the cotyledons resulting in more rapid water imbibition – a feature implicated in TVC (Mazibuko, 2003).

The activity of Imbali PME was relatively high in extracts from cotyledons at dormancy and R6, in contrast to the near absence of activity in the more resistant cultivars at dormancy, and moderate activity at R6. The presence of the enzyme in dormant cotyledons of Tongaat and Tokai (as shown by the electrophoresis gels in Figures 4.6, 4.7, 4.8), albeit inactive, suggests that the Imbali PME has post-translational elements that ensure its activity throughout the plant's vegetative state. The PMEs of the more resistant cultivars, on the other hand, may have a more rigid regulatory mechanism that limits their activities to the narrow period between germination and pod development. For all the varieties PME activity was optimal at R4 – the stage where seeds (and thus cotyledons) are developing. At this stage

PME activity is necessary since pectin de-esterification is necessary for wall modification to accommodate building blocks for the expanding organ.

As already mentioned, the cotyledons of TVC afflicted plants at dormancy exhibit cracks when imbibed in water. In view of this evidence, this study proposes that it is the activity of the enzyme/s at the R4 stage that is crucial in the development of TVC. In contrast to PME, PG activity at the R4 stage differed among the varieties and was coincident with each variety's susceptibility to TVC. This provides evidence that the activity of PG is the direct cause of structural weakness in TVC afflicted cotyledons, and the role of PME is indirect.

In view of the fact that a synergistic relationship exists among the various enzymes in the group of pectinolytic enzymes, zymogram experiments were conducted to characterize the active enzyme in the reaction (Fig. 4.2). The molecular weights of the active bands were within the range of plant PMEs (~30kDa) and PGs (~45kDa) listed in enzyme databases. High enzyme activities were reflected in the electrophoresis gels by thicker bands, indicating enzyme activity was determined by enzyme expression levels *in vivo*. This is of particular importance since it allows for rapid diagnosis of seed batches in the laboratory thus enabling the discarding of TVC prone bean lines.

References

Gross KC (1982) A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. HortScience **17**: 933-934

Hagerman AE, Austin PJ (1986) Continuous spectrophotometric assay for plant methyl esterase. Journal of Agricultural and Food Chemistry **34**: 440-444

Johansson K, El-Ahmad M, Friemann R, Jomvall H, Markovich O, Eklund H (2002) Crystal structure of plant pectin methylesterase. FEBS Letters **514**: 243-249

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680–685

Mazibuko TG (2003) Calcium regulation of water absorption to alleviate cotyledon cracking in green beans (*Phaseolus vulgaris* L.). MSc thesis. University of Natal, Pietermaritzburg, South Africa

Micheli F, Sundberg B, Goldberg R, Richard L (2000) Radial distribution pattern of pectin methylesterases across the cambial region of hybrid aspen at activity and dormancy. Plant Physiology **124**: 191-199

Pilatzke-Wunderlich I, Nessler C L (2001) Expression and activity of cell-wall-degrading enzymes in the latex of opium poppy, *Papaver somniferum* L. Plant Molecular Biology **45**: 567-576

Ren C, Kermode AR (2000) An increase in pectin methyl esterase activity accompanies dormancy breakage and germination of yellow cedar seeds. Plant Physiology **124**: 231-242

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

The cracking of cotyledons in green bean is a factor that contributes to lowered crop yield, thus limiting the availability of a major nutrient source to many societies in the under-developed world. The need exists therefore to identify and breed lines that are resistant to the condition in order to improve crop yield. This study characterized TVC at the physiological, biochemical and molecular level in the endeavour to find a means of identifying TVC susceptible *Phaseolus vulgaris* cultivar lines.

The cracking of bean cotyledons probably results when the cell walls cannot withstand the tensile stresses caused by water imbibition during germination. Wall coherence is maintained mainly by the pectin matrix in the middle lamella into which the cellulosic fibril network is embedded (Cosgrove, 1997). The strength of the pectin matrix is in itself dependent on cross-linkages between its polymers via (mostly) calcium cations (Marschner, 1995). Thus the amount of Ca^{++} in structurally impaired cotyledons may be expected to be low. This observation was made in this study, with the calcium content being inversely proportional to the incidence of TVC observed in the field.

It has also been observed that *P. vulgaris* varieties not differing significantly in wall Ca^{++} content may exhibit different levels of susceptibility to TVC. This can be attributed to their having different inherent qualities that confer different threshold levels to the build up of turgor pressure. Rapid water imbibition may, through mechanical forces, cause breakage of the cell walls. The rate of water imbibition is itself determined by (among others) the amount of water-soluble proteins and initial water content of the cotyledons (Hsu *et al.*, 1983). If sufficient water content is present, the proteins dissolve and result in a lower water potential within the cotyledonal cell wall, which in turn results in a more rapid uptake of water leading to wall rupture. In this study, the Imbali and Tokai TVC susceptible cultivars may have

had more water-soluble proteins in their constituent, as may be seen from their changing protein profiles after water treatment in Figs. 4.3 and 4.5 as opposed to the unchanging (resistant) Tongaat variety (Fig. 4.4).

In addition to the above, the degree of methylesterification is suggested to be a function of cell wall strength (Boccara and Chatain, 1989). Addition of methyl groups to wall components protects the cell wall structure from degradation by the host of cell wall modifying enzymes found in the plant cell. The methyl groups attach to cleavage sites thus protecting the polysaccharide polymers. The three examined varieties were in agreement with this assertion as well, with the degree of methylation being co-incidental with the degree of sensitivity to TVC of the respective varieties. The findings of this study lend compelling evidence that compromised wall integrity is the main physiological cause of TVC.

The activity of PME was similar for all cultivars at all growth stages examined. PME activity reached a peak between the VC and R4 stages of development then dropped as the plant reached R6. At these stages PME is involved in the processing of pectin for cell elongation (incorporation of building blocks) in order for the cotyledons to enlarge. Outside this period PME activity was absent. No correlation was found however between PME activity and TVC incidence in any of the cultivars.

The highly TVC-susceptible Imbali variety showed PG activity in all the growth stages investigated. The intermediate Tokai exhibited activity at VC and R4, while in the resistant Tongaat PG activity was only detected in the VC stage. The absence of Tongaat PG activity outside the VC stage is in agreement with the assertion that high PG activity at crucial stages where wall modification and expansion is taking place is the predetermining cause of TVC. At these fragile stages the building blocks may not be incorporated at a sufficient rate to replace the cleaved galacturonic acid residues, thus resulting in weak structures. The lowered resistance of the intermediate Tokai may be as a result of the presence of PG activity at the R4

stage of growth – an essentially anabolic phase where the seeds/cotyledons are formed.

Zymogram experiments allowed for the identification of a ~45kDa protein with PG-like properties (protein responded to PG zymogram conditions) and a ~30kDa molecule with PME properties. Expression levels of the molecules in electrophoresis gels also corresponded with estimated enzyme activity in biochemical tests.

The one procedure that is used to limit the incidence of TVC in the field has been the enhancement of seeds with calcium salts. While calcium nitrate is often recommended because it is readily available to the plant, there was no statistically significant difference between the three calcium salts. No difference in efficacy of calcium uptake was observed according to method of enhancement (priming or coating) although primed samples had significantly lowered germination potential and there was higher seedling abnormality than in the coated variety.

The findings in this project are that PG remains the prime candidate for the causal agent of TVC. However it should be taken into account that physiological disorders seldom have a single origin. Often various agents act in concert to bring about the phenotype. Also, questions arise from the observations noted above: Is the observed enzyme activity and TVC occurrence a function of the enzyme's own intrinsic property, or do methylation and/or calcium linkages play the major role in determining whether the cotyledons develop cracks regardless of the nature of PG enzyme? In the absence of information on these issues, the present study proposes the use of molecular based diagnostics such as Western blotting to identify and breed out of the cultivar lines that have significant poygalacturonase activity outside the VC phase of growth.

References

Boccara M, Chatain V (1989) Regulation and role in pathogenicity of *Erwinia chrysantheni* 3937 pectin methylesterase. Journal of Bacteriology **171**: 4085-4087

Cosgrove, D.J. (1997). Assembly and enlargement of the primary cell wall in plants. Annual Review of Cell Developmental Biology **13**:171-202

Hsu KH, Kim CJ, Wilson LA (1983) Factors affecting water uptake by soybean during soaking. Cereal Chemistry **60**: 208-211

Marschner H (1995) Mineral nutrition of higher plants, Ed 2. Academic Press, New York, pp 285-299

APPENDICES

Appendix 1. Field trial layout of plots of bean cultivars with different calcium salt treatments. The experimental line (seeds planted 10cm apart) was flanked by untreated lines so as to limit the effects of fluctuations in the environment. A gap of 50cm was left between adjacent plots, and a space of 1m left between blocks.

1. *Imb*P[Cl]^{10}	14. *Tong*C[Cl]^{100}	27. *Tok*C[S]^{100}	40. *Imb*C[Cont]	53. *Tong*C[Cl]^{100}	66. *Tok*C[S]^{100}	79. *Imb*P[Cl]^{10}	92. *Tong*C[Cl]^{100}	105. *Tok*C[S]^{100}
2. *Tong*P[Cl]^{10}	15. *Tok*C[Cl]^{100}	28. *Imb*P[N]^{10}	41. *Tong*C[Cont]	54. *Tok*C[Cl]^{100}	67. *Imb*P[N]^{10}	80. *Tong*P[Cl]^{10}	93. *Tok*C[Cl]^{100}	106. *Imb*P[N]^{10}
3. *Tok*P[Cl]^{10}	16. *Imb*P[S]^{10}	29. *Tong*P[N]^{10}	42. *Tok*C[Cont]	55. *Imb*P[S]^{10}	68. *Tong*P[N]^{10}	81. *Tok*P[Cl]^{10}	94. *Imb*P[S]^{10}	107. *Tong*P[N]^{10}
4. *Imb*P[Cont]	17. *Tong*P[S]^{10}	30. *Tok*P[N]^{10}	43. *Imb*P[Cl]^{10}	56. *Tong*P[S]^{10}	69. *Tok*P[N]^{10}	82. *Imb*[H2O]	95. *Tong*P[S]^{10}	108. *Tok*P[N]^{10}
5. *Tong*P[Cont]	18. *Tok*P[S]^{10}	31. *Imb*P[N]^{100}	44. *Tong*P[Cl]^{10}	57. *Tok*P[S]^{10}	70. *Imb*P[N]^{100}	83. *Tong*[H2O]	96. *Tok*P[S]^{10}	109. *Imb*P[N]^{100}
6. *Tok*P[Cont]	19. *Imb*P[S]^{100}	32. *Tong*P[N]^{100}	45. *Tok*P[Cl]^{10}	58. *Imb*P[S]^{100}	71. *Tong*P[N]^{100}	84. *Tok*[H2O]	97. *Imb*P[S]^{100}	110. *Tong*P[N]^{100}
7. *Imb*P[Cl]^{100}	20. *Tong*P[S]^{100}	33. *Tok*P[N]^{100}	46. *Imb*P[Cl]^{100}	59. *Tong*P[S]^{100}	72. *Tok*P[N]^{100}	85. *Imb*P[Cl]^{100}	98. *Tong*P[S]^{100}	111. *Tok*P[N]^{100}
8. *Tong*P[Cl]^{100}	21. *Tok*P[S]^{100}	34. *Imb*C[N]^{10}	47. *Tong*[PCl]^{100}	60. *Tok*P[S]^{100}	73. *Imb*C[N]^{10}	86. *Tong*P[Cl]^{100}	99. *Tok*P[S]^{100}	112. *Imb*C[N]^{10}
9. *Tok*P[Cl]^{100}	22. *Imb*C[S]^{10}	35. *Tong*C[N]^{10}	48. *Tok*P[Cl]^{100}	61. *Imb*C[S]^{10}	74. *Tong*C[N]^{10}	87. *Tok*P[Cl]^{100}	100. *Imb*C[S]^{10}	113. *Tong*C[N]^{10}
10. *Imb*C[Cl]^{10}	23. *Tong*C[S]^{10}	36. *Tok*C[N]^{10}	49. *Imb*C[Cl]^{10}	62. *Tong*C[S]^{10}	75. *Imb*C[Cl]^{10}	88. *Imb*C[Cl]^{10}	101. *Tong*C[S]^{10}	114. *Imb*C[Cl]^{10}
11. *Tong*C[Cl]^{10}	24. *Tok*C[S]^{10}	37. *Imb*C[N]^{100}	50. *Tong*C[Cl]^{10}	63. *Tok*C[S]^{10}	76. *Imb*C[N]^{100}	89. *Tong*C[Cl]^{10}	102. *Tok*C[S]^{10}	115. *Imb*C[N]^{100}
12. *Tok*C[Cl]^{10}	25. *Imb*C[S]^{100}	38. *Tong*C[N]^{100}	51. *Tok*C[Cl]^{10}	64. *Imb*C[S]^{100}	77. *Tong*C[N]^{100}	90. *Tok*C[Cl]^{10}	103. *Imb*C[S]^{100}	116. *Tong*C[N]^{100}
13. *Imb*C[Cl]^{100}	26. *Tong*C[S]^{100}	39. *Tok*C[N]^{100}	52. *Imb*C[Cl]^{100}	65. *Tong*C[S]^{100}	78. *Tok*C[N]^{100}	91. *Imb*C[Cl]^{100}	104. *Tong*C[S]^{100}	117. *Tok*C[N]^{100}
Block1			**Block2**			**Block3**		

Key: *Cultivar* Treatment [Salt] $^{\text{salt concentration}}$; where cultivar *Imb*= Imbali, *Tong*= Tongaat, and *Tok*= Tokai; treatment P= primed and C= coated; salt [Cl]= $CaCl_2$, [S]= $CaSO_4$, [N]= $Ca(NO_3)_2$; superscripts 10 and 100= salt concentrations of 10mM and 100mM respectively. Cont= controls and were distilled water for the priming experiments, and salt-less primer for the coating experiments.

Appendix 2. Analysis of variance for efficacy of seed calcium enhancement

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	2	0.47507	0.23754	11.16	0.023
Treatment	1	0.02668	0.02668	1.25	0.326
Salt	2	0.02521	0.01260	0.59	0.595
Level	1	0.03361	0.03361	1.58	0.277
Cultivar.Treatment	2	0.00774	0.00387	0.18	0.840
Cultivar.Salt	4	0.15018	0.03754	1.76	0.298
Treatment.Salt	2	0.14484	0.07242	3.40	0.137
Cultivar.Level	2	0.05077	0.02539	1.19	0.392
Treatment.Level	1	0.05921	0.05921	2.78	0.171
Salt.Level	2	0.00241	0.00120	0.06	0.946
Cultivar.Treatment.Salt	4	0.11084	0.02771	1.30	0.402
Cultivar.Treatment.Level	32	0.10971	0.05485	2.58	0.191
Cultivar.Salt.Level	4	0.14381	0.03595	1.69	0.312
Treatment.Salt.Level	2	0.07644	0.03822	1.80	0.278
Residual	4	0.08514	0.02129		
Total	35	1.5016			

Appendix 3. ANOVA tables for seed vigour analysis

A. Analysis of variance for germination potential.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	2	136.1	68.1	0.31	0.736
Treatment	1	4355.6	4355.6	20.10	<.001
Cultivar.Treatment	2	1019.4	509.7	2.35	0.137
Residual	12	2600.0	216.7		
Total 17 8111.1					

B. Analysis of variance for abnormal seedlings

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	2	1956.0	978.0	4.14	0.043
Treatment	1	7184.0	7184.0	30.41	<.001
Cultivar.Treatment	2	74.1	37.1	0.16	0.857
Residual	12	2835.3	236.3		
Total 17 12049.4					

Appendix 4. Analysis of variance for cultivar susceptibility to TVC

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	2	0.5355	0.2677	1.56	0.226
Residual	33	5.6727	0.1719		
Total	35	6.2081			

Appendix 5. Analysis of variance for cultivar yield

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	2	64.44	32.22	1.32	0.281
Residual	33	807.02	24.46		
Total	35	871.46			

Appendix 6. Regression statistics for the effect of TVC on yield

A. Effect of Imbali TVC incidence on yield

r	0.743983
r^2	0.553511
Adjusted r^2	0.508863
Standard Error	0.362449
Observations	12

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.628582	1.628582	12.39699	0.00553
Residual	10	1.313691	0.131369		
Total	11	2.942273			

B. Effect of Tongaat TVC incidence on yield

r	0.284244
r^2	0.080795
Adjusted r^2	-0.01113
Standard Error	0.618532
Observations	12

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.336276	0.336276	0.878964	0.370576
Residual	10	3.82582	0.382582		
Total	11	4.162096			

C. Effect of Tokai TVC incidence on yield

r	0.043424
r^2	0.001886
Adjusted r^2	-0.09793
Standard Error	0.589954
Observations	12

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.006575	0.006575	0.018892	0.893404
Residual	10	3.480458	0.348046		
Total	11	3.487033			

Appendix 7. Regression statistics for effect of cotyledonal calcium content on TVC

A. Effect of Imbali calcium on TVC

r	0.082123
r^2	0.006744
Adjusted r^2	-0.09258
Standard Error	0.744075
Observations	12

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.037592	0.037592	0.067899	
Residual	10	5.536482	0.553648		
Total	11	5.574074			

B. Tongaat calcium on TVC

r	0.07884
r^2	0.006216
Adjusted r^2	-0.09316
Standard Error	1.034346
Observations	12

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.066917	0.066917	0.062546	0.807578
Residual	10	10.69871	1.069871		
Total	11	10.76563			

C. Tokai calcium on TVC

r	0.254762
r^2	0.064904
Adjusted r^2	-0.02861
Standard Error	0.991666
Observations	12

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.682564	0.682564	0.694085	0.42423
Residual	10	9.83401	0.983401		
Total	11	10.51657			

Appendix 8. Hoagland's nutrient solution

Macromolecules		Micromolecules	
Compound	Concentration (M)	Compound	Mass ($g.L^{-1}$ dist H_2O)
$Ca(NO_3)_2.4H_2O$	1.0	$MnCl_2.4H_2O$	1.81
KNO_3	1.0	H_3BO_3	2.86
$MgSO4.7H_2O$	1.0	$CuSO_4.5H2O$	0.08
KH_2PO_4	1.0	$H_2MoO_4.H_2O$	0.09
Ferric citrate	0.007		

Appendix 9. Regression statistics of polygalacturonase (PG) at different stages of development

A. PG activity regression statistics at dormancy

	Imbali	Tongaat	Tokai
r	0.959	0.261	0.625
r^2	0.920	0.068	0.391
F significance	0.002	0.618	0.184

B. PG activity regression statistics at VC stage

	Imbali	Tongaat	Tokai
r	0.895	0.891	0.877
r^2	0.801	0.795	0.768
F significance	0.016	0.010	0.022

C. PG activity regression statistics at R4 stage

	Imbali	Tongaat	Tokai
r	0.874	0.584	0.834
r^2	0.765	0.341	0.695
F significance	0.023	0.224	0.039

D. PG activity regression statistics at R6 stage

	Imbali	Tongaat	Tokai
r	0.924	0.430	0.738
r^2	0.853	0.185	0.545
F significance	0.010	0.394	0.094

Appendix 10. Regression statistics of pectin methylesterase (PME) activity at different stages of development

A. PME activity regression statistics at dormancy

	Imbali	Tongaat	Tokai
r	0.549	0.080	4.75×10^{-8}
r^2	0.301	0.006	2.26×10^{-15}
F significance	<< 0.05	<< 0.05	<< 0.05

B. PME activity regression statistics at VC stage

	Imbali	Tongaat	Tokai
r	0.888	0.893	0.980
r^2	0.788	0.798	0.960
Significance F	<< 0.05	<< 0.05	<< 0.05

C. PME activity regression statistics at R4 stage

	Imbali	Tongaat	Tokai
r	0.900	0.985	0.978
r^2	0.811	0.970	0.957
Significance F	<< 0.05	<< 0.05	<< 0.05

D. PME activity regression statistics at R6 stage

	Imbali	Tongaat	Tokai
r	0.804	0.918	0.803
r^2	0.646	0.844	0.644
Significance F	<< 0.05	<< 0.05	<< 0.05

Appendix 11. Table of correlation coefficients

Degrees of Freedom	Probability, *p*		
	0.05	**0.01**	**0.001**
1	0.997	1.000	1.000
2	0.950	0.990	0.999
3	0.878	0.959	0.991
4	0.811	0.917	0.974
5	0.755	0.875	0.951
6	0.707	0.834	0.925
7	0.666	0.798	0.898
8	0.632	0.765	0.872
9	0.602	0.735	0.847
10	0.576	0.708	0.823
11	0.553	0.684	0.801
12	0.532	0.661	0.780
13	0.514	0.641	0.760
14	0.497	0.623	0.742
15	0.482	0.606	0.725
16	0.468	0.590	0.708
17	0.456	0.575	0.693
18	0.444	0.561	0.679
19	0.433	0.549	0.665
20	0.423	0.457	0.652
25	0.381	0.487	0.597
30	0.349	0.449	0.554
35	0.325	0.418	0.519
40	0.304	0.393	0.490
45	0.288	0.372	0.465
50	0.273	0.354	0.443
60	0.250	0.325	0.408
70	0.232	0.302	0.380
80	0.217	0.283	0.357
90	0.205	0.267	0.338
100	0.195	0.254	0.321