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DEVELOPMENT OF AN IN VITRO SELECTION TECHNIQUE OF POTATO CULTIVARS FOR IMPROVED RESISTANCE TO FUSARIUM OXYSPORUM

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DEVELOPMENT OF AN *IN VITRO* SELECTION TECHNIQUE OF POTATO CULTIVARS FOR IMPROVED RESISTANCE TO *FUSARIUM OXYSPORUM*

BY

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Dedicated to my parents, Natie and our children

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1. INTRODUCTION

Despite the existence of substantial breeding programmes, the most widely grown potato varieties in South Africa continue to be the old, established ones. Although these potato cultivars have very good qualities, they lack adaptation to some environmental factors and resistance to disease. These factors can be responsible for a decline in yield, quality and market value. An option in addressing these problems would be to improve a popular variety rather than creating a new one. Callus and plant cell cultures have provided a new and exciting option for increased genetic variability, relatively rapidly and without sophisticated technology.

Plant tissue culture has in recent years attracted growing interest from plant pathologists and breeders aiming for disease resistance, since it could meet the requirements for screening a large number of individuals in a small space (Chawla & Wenzel, 1987a).

Meyer (1967) found that *F. oxysporum* represented 80 - 90% of the total mycobiota of the rhizosphere of several agricultural crops. *Fusarium oxysporum* causes dry rot, stem-end rot and wilt of potatoes (*Solanum tuberosum*) (Nelson *et al.*, 1981). Because of the high frequency and economic importance of *Fusarium*, it is important to have potato cultivars and breeding lines resistant to these diseases. Fusarium wilt and stem-end rot are a problem under dry land conditions (A. Visser, personal communication). A high percentage of the potatoes in South Africa are cultivated under dry land conditions. Fusarium dry rot is a post harvest disease and a problem when potatoes are stored after harvesting, prior to the next planting season.

On a Potato Breeding Indaba held on the 11th of June 1991 at the ARC-Roodeplaat, the potato breeders and pathologists felt that screening for Fusarium resistance is important and that an effective screening method is essential. It was stated that cultivars or breeding lines with improved Fusarium resistance were needed, but these improved cultivars and/or breeding lines should not differ agronomically from their breeding parents. Conventional breeding strategies are time-consuming. It may be possible to shorten the process, by

developing a model, using cell biological techniques, by which improved resistance of potato cultivars and breeding lines to *F. oxysporum* f. sp. *tuberosi* can be obtained. At the meeting it was decided that an effective *in vitro* screening and selection technique needed to be developed for South African potato cultivars. It was meant to be the first model of it's kind in South Africa.

Fungi of the genus *Fusarium* are known to produce biologically active secondary metabolites displaying phytotoxicity in biotests on plants, but their role in pathogenesis was not quite clear (Hartman-Mitchell *et al.*, 1983; Scheffer, 1983). Selection for resistance to crude filtrates of pathogens *in vitro* may yield resistant plants, although it is not known exactly which toxins are present in the filtrates or what role they play in pathogenesis (Behnke, 1980a, b; Sacristan, 1982). In *Fusarium* there are over 100 known toxic metabolites which structurally belong to the group of the trichothecenes and zearalenone (Mirocha & Christensen, 1986). The level of knowledge on the role of these toxins in pathogenesis and the other physiological and biochemical bases of interaction of *Fusarium* with plants is limited.

Not all cell lines stably resistant to *Fusarium* spp. filtrate, regenerated plants with increased resistance to *Fusarium in vivo.* The same phenotype, i.e. resistance to toxic filtrates, may be due to different genetic or possibly epigenetic changes in different lines. Daubt (1986) found that in selection for resistance against a crude pathogen filtrate one must isolate as many resistant cell lines as possible, since many of them may have been selected for resistance to components other than the putative toxin, but if enough are generated, some may carry the desired resistance.

A question frequently discussed is the effect of the toxic filtrates used for selection on changes in ploidy of the regenerated plants. Hartman *et al.*, (1984a,b) found polyploid plants with a greater *in vivo* resistance among regenerants from calli resistant to filtrate. The fact that the effect of gene dose may play a role in the increased resistance to pathogens is also mentioned by Latudedada & Lucas (1983). In the experiments with alfalfa a low level of resistance to *Verticillium albo-atrum* in parents was simply amplified

in polyploid somaclones. Arcioni *et al.*, (1987) found increased ploidy among alfalfa regenerants after selection for *F. oxysporum* resistance. Apart from the length of action of the toxic filtrates the initial genotype used for selection also seems to play a role. As is shown by Alicchio *et al.* (1984) in *Solanum melongena* (eggplant), toxins of pathogens may affect chromosome number in cultivated cells by interacting with cell genotypes. Binarová *et al.*, (1990) used large scale selection in which they managed to obtain some alfalfa plants with increased resistance to *Fusarium* species. Hartman et al., (1984a,b) and Arcioni et al., (1987) selected callus cultures of alfalfa on a smaller scale, but achieved a higher number of resistant regenerated plants. This may be due to the different genetic backgrounds of the initial plant genotypes used for selection. Other authors indicated that the desired traits cannot be derived from any source of parental material and that in the case of disease resistance it would be better to start with the highest possible level of resistance in a desirable agronomic background (Daubt, 1986). In such material, resistance can be increased much more effectively by *in vitro* selection than by simple screening of somaclonal variants of regenerated plants. These results support the concept of using cell culture technology to increase resistance to *Fusarium* species, but they revealed the need to learn more about the biochemical and physiological bases of the interaction of pathogen *versus* plant *in vivo* and in cell culture *in vitro*.

Phytotoxins have been recognised as useful tools for the induction and selection of disease resistant plants by using tissue culture (Branchard, 1984; Daubt, 1984; Wenzel, 1985). Wheeler & Luke (1955) first used phytotoxins in resistance breeding. Correlation of resistance to a parasite and resistance to its toxins is a necessary prerequisite for such a use of phytotoxins. Kuo *et al.*, (1970), Byther & Steiner (1972) and Matern *et al.*, (1978) proved this correlation with some fungi. Phytotoxins are generally classified into two major groups : non-specific and host specific. The possible involvement of fusaric acid, a non-specific toxin, in disease development has been reviewed (Pegg, 1981). Fusaric acid (5n-2pyridine-carboxylic acid) is produced by many *Fusarium oxysporum* forma speciales (Davies, 1969; Prasad & Chaudhary, 1974; Mutert et al.,, 1981). Toyoda *et al.*, (1984) selected tomato calli resistant to fusaric acid and Wenzel et al., (1984) have used fusaric acid as a selective agent on barley. Many potato cultivars are susceptible

hosts to the pathogen *F. oxysporum* which may produce substantial quantities of fusaric acid in still cultures or in infected plants. Selection of tissue cultures resistant to fungal toxins has been investigated for several plant species and pathogens. For the production of resistant plants utilizing cell culture one of two methods may be used. (1) The selection of cell lines resistant to toxins produced by the pathogen *in vitro* followed by regeneration of plants from resistant cell lines and (2) screening regenerated plants from unselected cell cultures and identifying resistant somaclonal variants. The first method was successful in producing tobacco plants resistant to *Pseudomonas tabacci* (Wolf & Foster) Stevens (Carlson, 1973), potato plants resistant to *Fusarium oxysporum* Schlecht. f. sp. *tuberosi* (Weimer) Snyder and Hansen (Behnke, 1980a) and *Phytophtora infestans* (Mont.) De Bary (Behnke, 1979; 1980b), maize plants resistant to *Helminthosporium maydis* (Nishikado & Miyake) race T (Gengenbach & Green, 1975; Gengenbach *et al.*, 1977) Brettell *et al.*, 1980, *Brassica napus* L. plants resistant to *Phoma lingam* (Tode ex. Fr.) Desm. (Sacristan, 1982) and *Medicago sativa* L. resistant to *F. oxysporum* f. sp. *medicaginis* (Hartman et al., 1984a,b).

The second procedure, where potential somaclonal variants are screened, proved to be successful in identifying potato plants resistant to *Alternaria solani* (Ellis & Martin) Jones and Grout (Matern *et al.*, 1978) and to *P. infestans* (Sheppard *et al.*, 1980), sugarcane plants resistant to fiji disease, *Sclerospora sacchari* (T. Miyake) and *Drechslera sacchari* (Butl.) Subram and Jain (Heinz *et al.*, 1977) and maize plants resistant to *H. maydis* race T (Brettell *et al.*, 1979).

"Somaclonal variation" is the general term used for variation detected in plants derived from any form of cell culture (Larken & Scowcroft, 1981). This variability may have its origin in the somatic cells or it could be induced by the culture media used. This *in vitro* induction of mutations can be used in the upgrading and improving of existing cultivars. Somaclonal variation can help the breeder by reviving failed breeding lines or by allowing unexpected variation to be expressed. Although *in vitro* inductions of mutations cannot replace cross-breeding, it can be valuable in supporting breeding programmes (Ancora & Sonnino, 1987). Several successes have been accomplished with somaclonal variation in

potato. Larkin & Scowcroft (1981) found variation in compactness of growth habit, maturity date, tuber uniformity, tuber skin colour, photoperiod requirements and yield. It was also found that this variation was significant and stable.

Shahin & Spivey (1986) found that the extent to which *in vitro* selection can be exploited in this way will, however, depend on whether the recovered undesirable heritable traits could impede the breeding process. From their results on tomatoes it is clear that many undesirable traits were recovered besides the targeted one (*Fusarium* wilt resistance). Unless there is a method to sort out those undesirable changes, the use of *in vitro* selection will be cumbersome to the plant breeder. Nevertheless, *in vitro* selection has its advantage as a potential source of novel breeding material for traits otherwise unobtainable by conventional breeding methods.

Chawla & Wenzel (1987a) found with barley plants that the character selected for at the plant level was not expressed as a qualitative trait in all cases. Some selected plants showed only partial resistance, indicative of a more quantitative expression. This variability of regenerated plants in the trait selected for has also been found in rape seed for *Phoma lingam* resistance (Sacristan, 1982) and in wheat and barley for *Helminthosporium sativum* resistance (Chawla & Wenzel, 1987b). The variation may be explained by the strong selection pressure, which results in different types of mutation, acting at different stages of the reaction to a particular toxin or pathogen. This implies furthermore that genetic rather than epigenetic changes at the cellular level have taken place. It has to be checked by segregation experiments and the reaction of lines insensitive to the toxin has to be compared with their reaction to the pathogen (Chawla & Wenzel, 1987a).

Although the first reports concerning disease resistant mutants obtained through cell culture techniques date back to the 1970's, only a few of these plants have found practical utility in crop improvement. The reasons for this are many, but the lack of reliable resistance tests at the whole plant level and frequent sterility or other agronomically undesirable traits in the regenerants are probably the most important (Sacristan, 1986).

The most important limitation in the use of cell culture systems for induction and selection of disease resistant mutants is the difficulty in finding unequivocal indicators for plant resistance at cellular level. Identification and understanding of the mechanisms of resistance and inter-relationships among them will help in planning more efficient procedures designed to select disease resistant mutants in tissue cultured cells. The general technique used at present is based on the application of behavioural criteria, i.e. survival and growth under the influence of a selective agent.

In vitro approaches hold considerable potential for increasing the genetic diversity available to the breeder and hence would be of use in disease resistance breeding.

In vitro selection for improved resistance of potato cultivars to *Fusarium oxysporum* causing dry-rot implies the induction of variation in tissue cultures of the crop (i.e. callus cultures, cell suspension cultures, protoplast cultures) followed by selecting cells with desired traits at cellular level using a selective agent, preferably a fungal toxin. This method has been applied successfully for the first time by Gengenbach & Green (1975) for the selection of *Helminthosporium* resistant corn plants. Since then, this approach has been used frequently, also aimed at the introduction of *Fusarium* resistance in different crops (Behnke, 1980a,b; Hartman *et al.*, 1984a; Arcioni *et al.*, 1987; Chawla & Wenzel, 1987a). Despite so many efforts, the prospects of this method are still not clear. In many studies resistant material has been claimed to be selected, but in only very few cases those studies involve genetic analysis of the acquired resistance. Genetic analysis was performed by Shahin & Spivey (1986) who claimed to have selected monogenic *Fusarium* resistance in protoplast cultures of tomato using fusaric acid as selective agent. However, close consideration of their results raises the question whether cells selected for insensitivity for fusaric acid result in more *Fusarium* resistant plants than the non-selected controls. A further examination of the potential of *in vitro* selection for *Fusarium* resistance seems therefore necessary.

Three factors determine the success of *in vitro* selection. Firstly, the tissue culture techniques must be sufficiently developed. Secondly, the variation in the tissue cultures

must be broad enough to contain the cells with the desired characteristic. Thirdly, the selective agent applied must play an important role in the pathogenesis.

AIM

From the literature study it can be seen that although success with *in vitro* selection have been obtained in a few crops, an effective model for screening and selection against *Fusarium oxysporum* forma speciales for any specific crop has not yet been developed. To develop an effective model, the three factors discussed above should be in place for every crop.

The aim of this study was therefore to develop an effective *in vitro* selection technique to select potato somaclones or breeding lines with improved resistance to *Fusarium oxysporum*.

The work will be done in three phases:

a) The existing cultivars will be established *in vitro*. These *in vitro* plantlets will then be used as explant material for the induction of a callus phase. The callus will be propagated until white friable calli are obtained. These friable calli will subsequently be used as inoculum for cell suspensions. After establishing a good cell suspension, the cells will be plated. The plated cells will be developed into microcalli and these microcalli will then be greened and enlarged, before they are transferred to shoot regeneration medium. The shoots will be rooted before transplanting them into soil.

   Promising clones can already be selected in the test tube by discarding all the malformed plants. Plant regeneration is a selection process in itself, as only cells which possess the genetic ability to regenerate plants, are used. Because desirable somaclones can be identified in the test tube, it makes somaclonal variation much more efficient and cost effective.

In short, it can be said that the *in vitro* techniques for the South African potato cultivars will be established and optimized. It is important to develop an optimal *in vitro* system which could enable one to add selective agents (toxin, culture filtrates) to screen the potato cultivars for better resistance against *Fusarium oxysporum*. This was important, since one have to be sure that clones which perform poorly do so because of the selective agent and not because it is not optimally grown in culture. This phase addresses factor one, namely that tissue culture techniques must be sufficiently developed and is described in chapters 2,3, 4 and 5.

b) The role which the toxin plays in disease development (dry rot) in the potato cultivars will be determined and the pathogen will be characterized. This is described in chapters 6, 7 and 8.

c) The toxicity of *F. oxysporum* culture filtrate and fusaric acid on cell and callus cultures of different resistant and susceptible genotypes will be described in order to determine whether an eventual relationship exists between susceptibility of potatoes to *F. oxysporum*, fusaric acid and culture filtrates of *F. oxysporum*. This study may provide the tools to develop a model to select plants with improved resistance to other phytopathogenic fungi by selecting callus resistant to the congenial toxin (chapters 9, 10 and 11).

d) Regenerated potato plants will be tested for improved resistance to *F. oxysporum*. This programme is dependent on linkage to the conventional potato breeding programme. This linkage will ensure that the tissue culture material developed has the agronomic performance that is essential (chapter 12).

The potato cultivars used during the study were recommended by the researchers of the *Fusarium*-resistance breeding programme at ARC-Roodeplaat. The potato

cultivars 'Late Harvest', 'BP1', 'Vanderplank', 'Kimberley Choice' and 'Up-to-Date' will be used for the study. 'Late Harvest' will be included since it is the cultivar with the best existing resistance against Fusarium dry rot in South Africa (Steyn *et al.*, 1991). 'BP1', 'Up-to-Date' and 'Vanderplank' are concidered to be moderately resistant and 'Kimberley Choice' is the most susceptible cultivar. These cultivars are of the more popular cultivars and are included as standard cultivars in the potato breeding programme in South Africa. Of all the commercial seed potato cultivars grown in South Africa 46 % is 'BP1', 19 % 'Up-to-Date' and 7 % 'Vanderplank' (Nortje, 1997).

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2. OPTIMIZATION OF THE *IN VITRO* PRODUCTION OF POTATOES IN SOUTH AFRICA*

INTRODUCTION

Micropropagation is a reality in the potato industry in South Africa. The use of micropropagation in potato seed production is a common practice world-wide (Lam, 1975; Roest & Bokelman, 1976; Cardi et al.,, 1993). In most instances the use of micropropagation has led to a reduction in the number of generations needed in the field (Cardi et al., 1993). Potato plants grown *in vitro* are widely used as a source for the production of micro- and minitubers (Hussey & Stacey, 1984) . The greatest need is for a tissue culture system in which such plants can be produced cost and labour effectively in a commercial setup. Where micro- or minitubers can be used to produce a commercial crop after only one multiplication step on an economically justifiable scale, it would certainly have a significant impact on the potato seed production industry.

The value of the production of microtubers has recently been recognised. Microtubers can be used successfully to produce minitubers, but can also be utilised in various other ways. It can be used for distribution of potato plant material nationally as well as internationally (Hussey & Stacey, 1984). Microtubers are produced from plantlets tested for pathogens and will therefore comply easily with international quarantine requirements. It can be exported to neighbouring countries as a basis for further propagation, may be utilised in experiments aimed at genetically engineering superior potatoes and can be used effectively for the *in vitro* conservation and maintenance of large germplasm collections (especially long-term storage) (Joerdens-Roettger, 1987). The need is to develop a method which will be applicable to a wide range of genotypes, has a short tuberization induction period and requires minimal manipulation of plant material in order to ensure cost and labour effectivity.

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Cytokinins and growth regulators are believed to have strong promotive effects on tuberization and to constitute part of the tuberization stimulus, either alone or in combination with other subtance(s) (Palmer & Smith, 1969, 1970; Forsline & Langille, 1976; Leclerc *et al.*, 1994). However growth regulators failed to induce tuberization when the sucrose supply was inadequate (Harmey *et al.*, 1966; Leclerc *et al.*, 1994). High sucrose concentration may be the only compound necessary for induction of microtubers (8 % in stead of 2 %) (Ewing, 1985, 1990; Forti *et al.*, 1991; Ranalli *et al.*, 1994).

It has also been stated that the use of growth retardants improved the microtuber formation by potato (Forti *et al.*, 1991; Harvey *et al.*, 1991; Leclerc *et al.*, 1994). The effect of growth regulators on tuberization depend on genotype. These effects can be of marked increase of the tuberization percentages and weights, but also of a decrease of the same parameters (Forti *et al.*, 1991; Leclerc *et al.*, 1994). Increasing the production of microtuber systems while reducing the medium complexity and cost is important to meet this goal. Experiments were conducted to evaluate the efficiency of chlorocholine chloride (CCC), BA and Alar as promoters of microtuberization of South African potato cultivars.

This study therefore focussed on the development of a tissue culture system for Potato South Africa in which plants, micro- and minitubers can be used to produce a commercial crop with reduced multiplication steps on an economically justifiable scale. Although done elsewhere in the world, this will be the first study of its kind in South Africa and on the main patato cultivars in South Africa.

MATERIALS AND METHODS

Plant Material

Virus-free plant material of three cultivars, 'Late Harvest' (LH), 'BP1' and 'Up-to-Date' (UTD) was obtained from the potato genebank at the ARC-Roodeplaat Vegetable and Ornamental Plant Institute.

Ten tubers of each were planted in plastic plant bags (120mm x 250mm) filled with sterilized soil and grown in a greenhouse. Stem tissue was dissected at a height of 150mm on four week old plants.

The stems were cut into segments, consisting of a nodal cutting containing one axillary bud and subtending leaf. The segments were then washed three times with distilled water. Heavily soiled material was rinsed under running tap water for an hour, before it was washed with distilled water. A 1% (v/v) 7X detergent solution, was added to reduce the surface tension in order to enhance the activity of the sterilant. A 0.1% sodium hypochloride (NaOCl) (v/v) solution was then added and stirred for ten minutes. The stem segments were subsequently sterilized in a mixture of 1% (v/v) NaOCl and one drop of Tween 20 for 15, 20 and 25 min respectively. The segments were then rinsed five times with sterile distilled water to ensure the complete removal of NaOCl.

Nutrient media, consisting of basic Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) supplemented with 0.01mg.dm^{-3} naphthaleneacetic acid (NAA), 0.1mg.dm^{-3} gibberellic acid (GA_3), 25g.dm^{-3} sucrose and 7g.dm^{-3} agar (Biolab Agar commercial Gel, Merck) as solidifying agent was used. The pH was adjusted to 5.5 with 1M sodium hydroxide (NaOH). Borosilicate glass test tubes containing 10cm^3 of nutrient media were used as culture vessels. The medium was sterilized in an autoclave at 121°C and a pressure of 103kPa for 20 minutes. The GA_3 was filter-sterilized by means of 0.22µm Millipore filters and added after the medium was autoclaved and allowed to cool.

After disinfection, plant parts were carefully trimmed by cutting away all bleached and dead tissue under sterile conditions in a laminar flow cabinet. The healthy stem segments were individually placed upright with the bottom cut end in the medium in the test tube. Some of the test tubes were sealed with alluminium lids (cap-o-tests), some were sealed with cap-o-tests with one layer of Parafilm and others had two layers of Parafilm. The cultures were incubated at 26°±1°C with a 16h photoperiod under cool white fluorescent tubes (55 µmol.m^{-2}.s^{-1}).

Nodal cuttings of the sterile *in vitro* plantlets were then subcultured until the required number of plantlets for callus initiation had been obtained. The incubative period was 4 weeks. A stock culture was maintained as a reserve in case of contamination.
A supply of shoot cultures of each cultivar was maintained by subculturing single stem nodes every 3 - 4 weeks. These were cultured in 16 h days at 24°C and a light intensity of 55µmol.$m^{-2}s^{-1}$.

Factors affecting the cost

To optimize the commercial *in vitro* production of potato plantlets five aspects are of importance :

- tissue culture containers
- tissue culture media
- explant density
- growth conditions.

Tissue culture containers

For the production of *in vitro* potato plants four tissue culture containers with different explant densities were tested (Table 2.1; Figure 2.7). The time of transplantation to and from tissue culture containers were measured. Three operators were used. The amount of medium needed for different containers as well as the space used in the growth chambers were determined (Table 2.1). The standard tissue culture container used by most laboratories in South Africa for potato micropropagation is a test tube (1 or 2 cm in diameter) (Fig. 2.7) although some have changed to bulk containers. (Plastic tubs, 250 cm^3) Nodal cuttings were inoculated into the different tissue culture containers.

Plastic tubs (500 mℓ) (Fig 2.7) were used for the production of microtubers on solid media and 250 mℓ Erlenmeyer flasks were used for production of microtubers in liquid cultures.

Tissue culture media

To determine the optimum tissue culture medium for production of *in vitro* potato plants, plastic tubs containing 40 *in vitro* explants were used. The number of nodes (axillary buds) per plant were counted weekly for each of the five media. Morphological differences between explants cultured in different media were noted as well as differences in stem length and internode distance. Twenty tubs per medium per cultivar were used. Five different tissue culture media were tested, namely:

1) Murashige and Skoog (MS) medium (Murashige & Skoog, 1962);
2) MS medium supplemented with 1.5 $mg.dm^{-3}$ silver thiosulphate;
3) MS medium supplemented with 0,3 $mg.dm^{-3}$ thiamine HCl, 0,01 $mg.dm^{-3}$ NAA and 0,1 $mg.dm^{-3}$ GA_3 (regeneration medium);
4) MS medium supplemented with 0,1 $mg.dm^{-3}$ GA_3 and 0,01 $mg.dm^{-3}$ NAA;
5) Philippine medium (Joerdens-Roettger, 1987).

For the production of microtubers five different tissue culture media in 250 cm^3 plastic tubs were evaluated :

1) MS medium supplemented with 8% sucrose (solid), 7 % agar; (Biolab Agar Commercial Gel, Merck).
2) MS medium supplemented with 0,4 $mg.dm^{-3}$ Alar and 5% sucrose, 7 % agar (Biolab Agar Commercial Gel, Merck) (solid) (Alar medium);
3) MS medium supplemented with 5 $mg.dm^{-3}$ BA, 500 $mg.dm^{3}$ chlorocholine chloride, 100 $mg.dm^{-3}\ell$ Inositol and 8% sucrose (solid) (CCC medium);
4) MS medium supplemented with 5 $mg.dm^{-3}$ BA, 500 $mg.dm^{3}$ chlorocholine chloride, 100 $mg.dm^{-3}$ Inositol and 8% sucrose (liquid) [Two phase production (Estrada *et al.*, 1986)];
5) MS medium supplemented with 8% sucrose (liquid).

A limited number of microtubers were produced on hormone-free medium. This correlated with results found by Belletti *et al.*, (1994). The two phase production of microtubers in liquid cultures described by Estrada *et al.*, (1986), proved to be more labour intensive, took more time and callus formation was a problem.

It was therefore decided to use the same media described by Estrada et al., (1986), but it was solidified with 0,8% Agar (Biolab Agar commercial Gel, Merck). This resulted in the fact that only medium 2 (Alar medium) and medium 3 (CCC medium) were tested in detailed experiments. The best medium was chosen according to the number of microtubers and mass of microtubers formed. Forty single nodes of each cultivar were transferred to the tuber CCC and Alar media for tuber initiation. Number of microtubers formed were counted and every tuber was weighed.

Tissue culture conditions

For the production of potato plants *in vitro* the plants were grown at 24°C with a 16 h day provided by 1 200 mm fluorescent tubes with a light intensity of 55 $\mu mol.m^{-2}s^{-1}$
For the production of microtubers the plants were grown at 5, 19 and 24°C with a 16 h day provided by 1 200 mm fluorescent tubes with a light intensity of 55 $\mu mol.m^{-2}s^{-1}$ or in total darkness.

Table 2.1 **Different tissue culture containers and explant densities with the quantity medium (dm^3) and space in growth chamber (m^2) needed for each**

Tissue culture container	Explant density (explants per container)	Medium needed to produce 200 explants (ℓ)	Space in growth chamber for 200 explants (m^2)
1. Test tube (1 cm diameter)	1	2	0,5 m^2
2. Test tube (2 cm diameter)	2	2	0,25 m^2
3. Test tube (2 cm diameter)	4	1	0,125 m^2
4. Screw cap bottle (250 mℓ) (50 mm diameter)	4	2,5	1 m^2
5. Screw cap bottle (250 mℓ) (50 mm diameter)	8	1,25	0,5 m^2
6. Screw cap bottle (250 mℓ) (50 mm diameter)	12	0,9	0,3 m^2
7. Plastic tub (500 mℓ) (110 mm diameter)	20	1	0,25 m^2
8. Plastic tub (500 mℓ) (110 mm diameter)	30	0,7	0,3 m^2
9. Plastic tub (500 mℓ) (110 mm diameter)	40	0,5	0,125 m^2
10. Plastic tub (500 mℓ) (110 mm diameter)	50	0,4	0,125 m^2
11. Plastic tub (500 mℓ) (110 mm diameter)	60	0,35	0,125 m^2

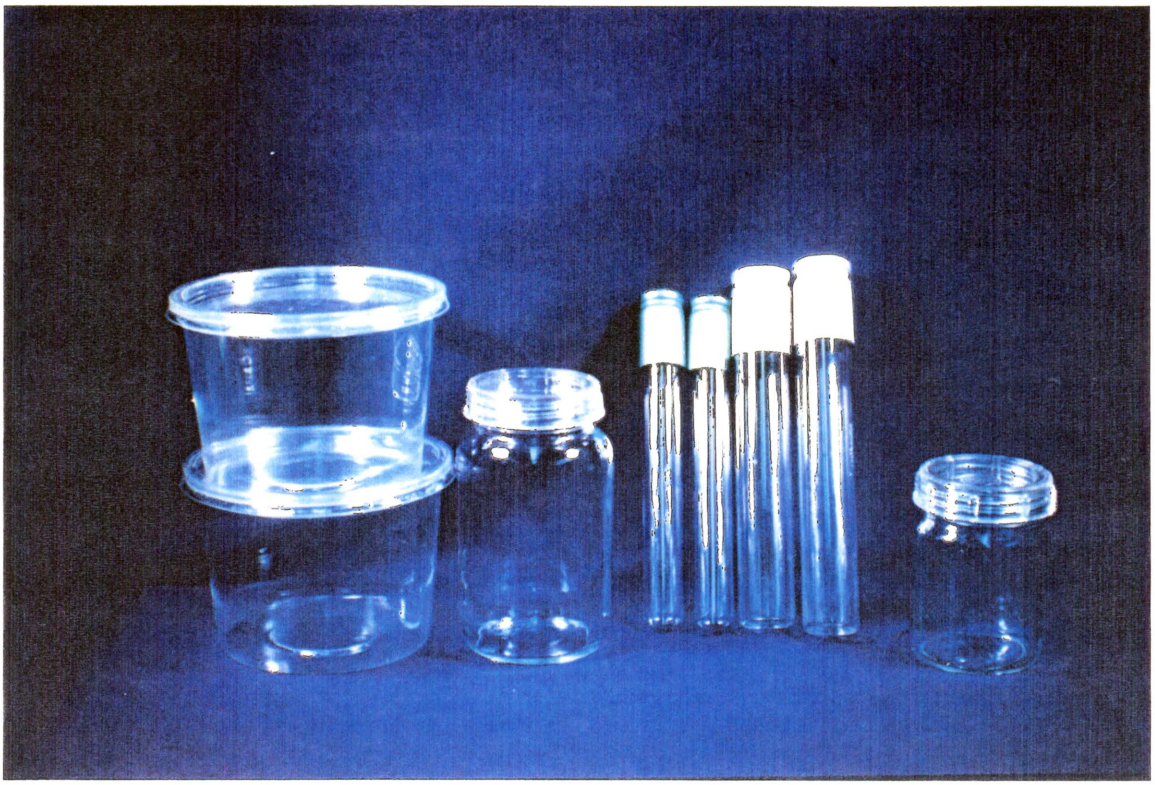

Figure 2.7: Tissue culture containers used for the *in vitro* production of potato plants (from left to right: plastic tubs, screw cap bottles (250 ml), test tubes (1 cm), test tubes (2 cm) screw cap bottle (100 ml) (not used).

RESULTS AND DISCUSSION

It is of great importance to determine the right duration for surface-sterilizing the explant material. Trial runs indicated that 25 min was too long. These stem segments were bleached white, which was an indication that the material was dead. The axillary buds were also damaged and could therefore not be used for initiating *in vitro* cultures. Only three shoots developed from these axillary buds (Table 2.2).

It was also found that a sterilization period of 15 min was too short. The material was not damaged, but because of the short period, many contaminants survived the treatment and contamination occurred within one week of incubation. Although plantlets developed from these cultures they could not be used in further experiments (Table 2.2). A period of 20 min for the surface sterilization of material was sufficient. All the dead material could carefully be cut away and contamination was minimal (Table 2.2).

The plantlets in test tubes with cap-o-tests as well as two layers of Parafilm, had thin hairy stems with small leaf blades. Perl *et al.*, (1988) attributed these morphological changes

to the build-up of ethylene in the tubes. The cultures covered with cap-o-tests and one layer of Parafilm, also had weak, elongated stems with small leafblades. Cultures with cap-o-tests but without Parafilm developed into normal healthy plants which could be used for the initiation of callus culture. Plants were therefore cultured in glass tubes with cap-o-tests but without parafilm in order to obtain plants. These cultures showed more contamination than with parafilm.

Table 2.2 Effect of duration of surface-sterilising of potato explant material on contamination rates

	TIME OF SURFACE-STERILISING (minutes)		
	15	**20**	**25**
Total number of explants inoculated	500	500	500
Total number of explants initiating shoots	107	463	3
Total number of explants contaminated	489	116	0

In vitro production of potato plants

Tissue culture container

Transplantation of plantlets from plastic tubs containing 50 or 60 plantlets was far less time-consuming than from any other container. The container and explant density had an influence on the speed of inoculation of the operators ($P < 0.001$) (Figure 2.1). Experience of operators may play a major role in the time needed for inoculation (Figure 2.1). Tukey's multiple range test indicated that there were statistically significant differences between time needed for inoculation of the different containers containing different numbers of explants ($P < 0,001$, $LSD_T = 0,096$) and between the time of inoculation of the different operators. The relationship between the time needed for inoculation of different containers with different explant densities and different operators declared the most variance. According to the time needed for inoculation it is less time

consuming using plastic tubs with 40, 50 or 60 explants than anyone of the other containers (Figure 2.1). Although there were no significant differences between the time needed to inoculate a shoot in plastic tubs containing 40, 50 or 60 plants (Figure 2.1), visual evaluation of *in vitro* plantlets four weeks after transplantation showed that *in vitro* plants in a plastic tub with 60 explants were hyperhydrated and showed symptoms of nutrient deficiency (Figure 2.8). Plantlets in a plastic tub containing 40 explants were looking the best. For logistical reasons (easier to count and calculate orders) it may be preferred to use plastic tubs containing 50 explants.

Plastic tubs containing 40 or 50 explants required less medium to produce 200 plants than that of test tubes. It needed about a quarter of the medium to produce 200 plants than that of test tubes and also much less than most of the other containers and explant densities (Table 2.1 and Figure 2.9). Another major advantage of plastic tubs containing 40 or 50 explants was that about a quarter of the space in the tissue culture growth room was required when compared to test tubes (Table 2.1). By using plastic tubs containing 40 or 50 explants, time, space and media can be utilised 75% more effectively than using the test tubes.

Figure 2.8 Visual evaluation of plantlets four weeks after transplantation
(A) Plastic tub containing 60 plantlets (hyperhydrated)
(B) Plastic tub containing 40 plantlets.

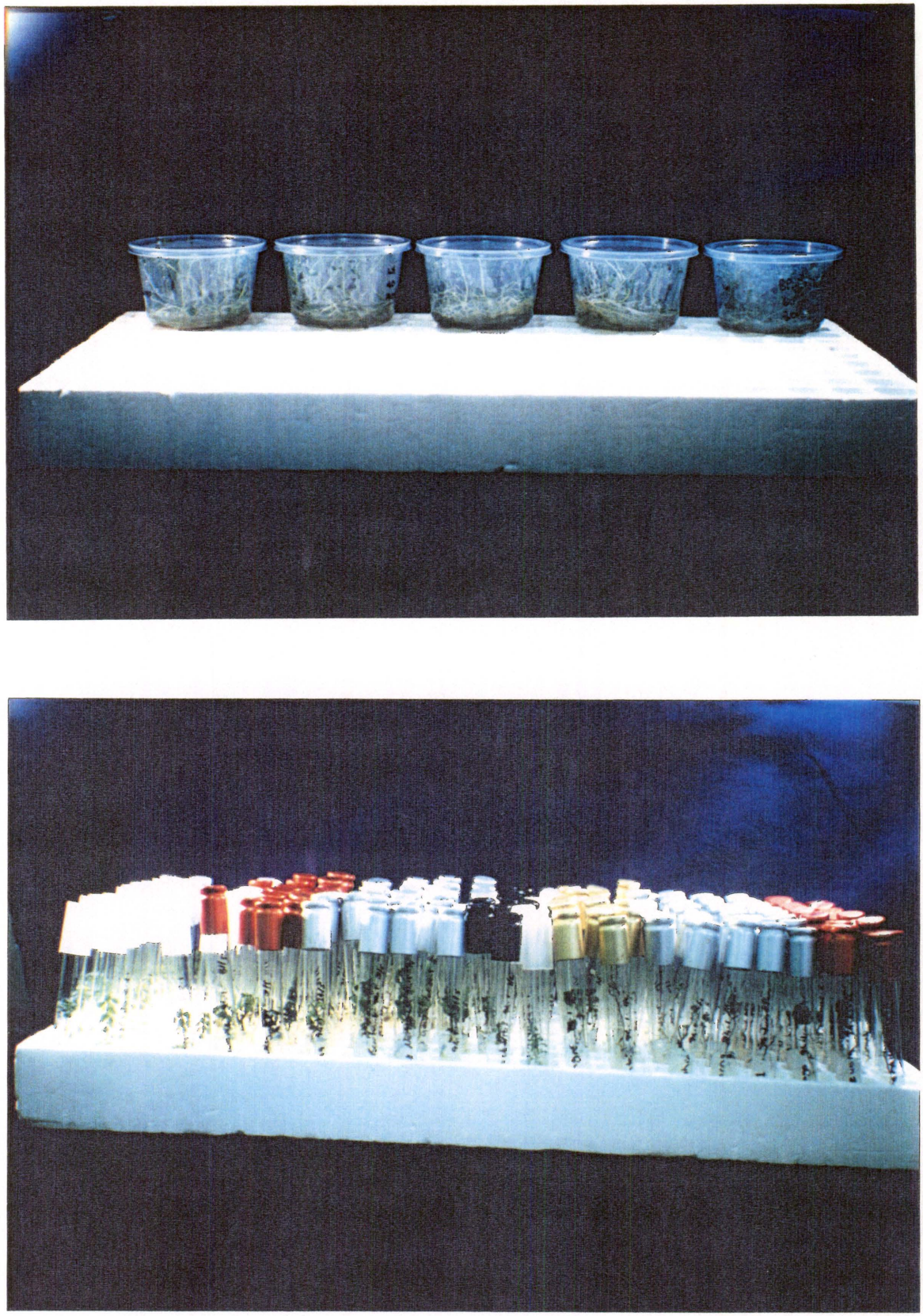

Figure 2.9 A: Plastic tubs containing 40 explants each (200 in total) displayed on seedling tray which normally contains 200 test tubes.
B: Seedling tray containing 200 test tubes.

Tissue culture medium

Time has an influence on the effect that the medium has on the number of axillary buds produced ($P < 0,001$) (Figure 2.2). There were statistical significant differences in the number of axillary buds produced by plants grown in the different media ($P < 0,001$), and the different weeks ($P < 0,001$). This illustrates that the variation in number of axillary buds can be explained by the differences in media and time (Figure 2.2). The number of axillary buds increased as the weeks increased (Figure 2.3). Plantlets showed serious symptoms of hyperhydricity and nutrient deficiency seven weeks after transplantation. Although plants developed more axillary buds up until week 8, the visual quality of the plants decreased from week 6. The plantlets cultured on MS supplemented with silver thiosulphate were shorter and more sturdy than plantlets grown in other media (Figure 2.10) The leaves were larger and darker green than those on the other media (Figure 2.10). This was an advantage when plants were transplanted to the greenhouse. Short, sturdy plants with large leaves tend to adapt more easily to external conditions. Although optimum numbers of axillary buds have not been obtained by the fourth week after inoculation, it is best to subculture plantlets between week 3 and 4 as plant vitality starts to decrease after week 5 (hyperhydricity and nutrient deficiency). During weeks 3 to 5 plants were in the logarithmic growth phase (Figure 2.2). MS medium supplemented with silver thiosulphate performed significantly better than the other four media whereas there were no significant differences between the MS and two other MS supplemented media: The Philipine medium (MS medium without vitamins) performed significantly poorer than any of the other media ($P < 0,001$) (Figure 2.4). Although MS medium supplemented with sliver thiosulphate was the optimum medium for the production of *in vitro* potato plants it is expensive and it is recommended that MS medium be used for rapid multiplication and that silver thiosulphate be added to the medium in the last multiplication step just before transplantation in the greenhouse, because the plants will be short and sturdy and will be able to adapt more easily to external conditions.

Figure 2.10: Late Harvest (left) and BP1 (right), plantlets cultured in MS medium supplemented with 1.5 mg.dm^{-3} silver thiosulphate.

In vitro production of microtubers

Microtuber production followed the pattern discussed by many authors. Microtubers began to appear 4 weeks after planting. Some were epigean and other hypogeous. Microtubers which developed inside the culture medium either grew from stolons directly grown inside the medium or were produced from shoots that only subsequently grew downwards. Generally one and sometimes two microtubers were formed per explant. A wide range of shapes were identified (round to elongated) and differences in surfaces (smooth or rough) and colour (yellow to green) were also recorded (Figure 2.11). This correlated with descriptions made by Ranalli et al., (1994). The number of tubers formed and their mass varied with the cultivar and the medium ($P < 0,001$). Most microtubers weighed between 20 and 60 mg.

Figure 2.11: Various shapes and surfaces of microtubers obtained from BP1.

CCC medium produced significantly more microtubers than Alar medium ($P < 0,001$), but there were no significant differences in the mass of the tubers produced on the two media. There were, however, significant differences between the mass of the microtubers of the different cultivars, 'Late Harvest' and 'BP1' produced tubers weighing significantly more than those of 'Up-to-Date' ($P < 0,001$) (Figure 2.5). 'Late Harvest' also produced significantly more microtubers than 'Up-to-Date' which in turn produced significantly more microtubers than 'BP1' ($P < 0,001$) (Figure 2.6). Although mass of microtubers is an important factor, the number of microtubers produced is more important and therefore it can be said that CCC medium was the best for the production of microtubers (Figure 2.12).

It was noted that microtubers were still viable after 21 months in culture (Figure 2.13). This is of major advantage for the maintenance of potato germplasm collection in South Africa. Preliminary results showed that at a temperature of 19 °C and total darkness, a high percentage of microtubers could be obtained. These results should be confirmed by more extensive research as well as dormancy of microtubers.

Figure 2.12: Microtubers produced on chlorocholine chloride (ccc) medium.

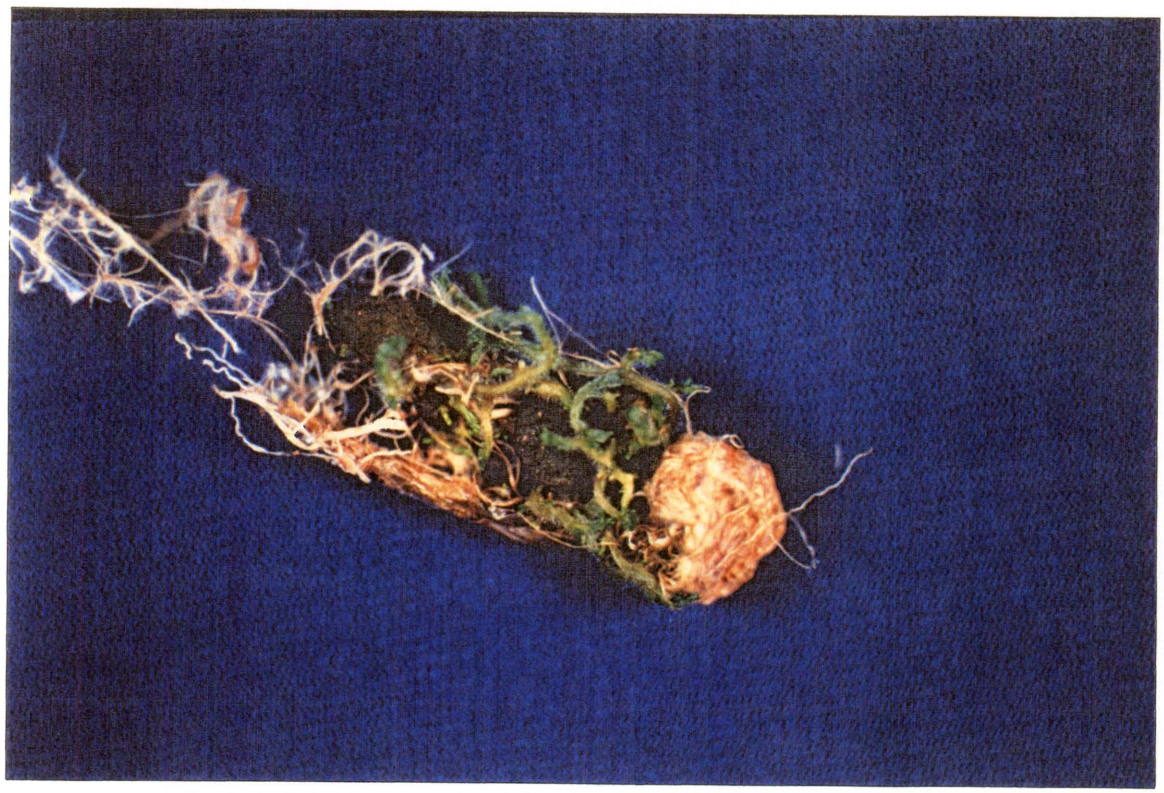

Figure 2.13: Microtubers of Late Harvest after 21 months in *in vitro* culture.

CONCLUSION

From the results obtained it is apparent that *in vitro* plants can be produced cost-effectively in a commercial set-up. Time, space and media can be used 75% more effectively, when 250 cm^3 plastic tubs are used in stead of 1 cm^3 test tubes. *In vitro* potato plants and microtubers can be produced more cost effectively. This will certainly have a significant impact on the potato seed production industry in South Africa.
Results obtained indicated that it will be possible to develop a schedulling programme for *in vitro* production of potato plants of the different cultivars in South Africa. This will enable laboratories to optimize their production time which will become more and more important since the number of role players is increasing and competition will increase and become a very important factor.

ACKNOWLEDGEMENT

We wish to thank Potato South Africa for funding this project.

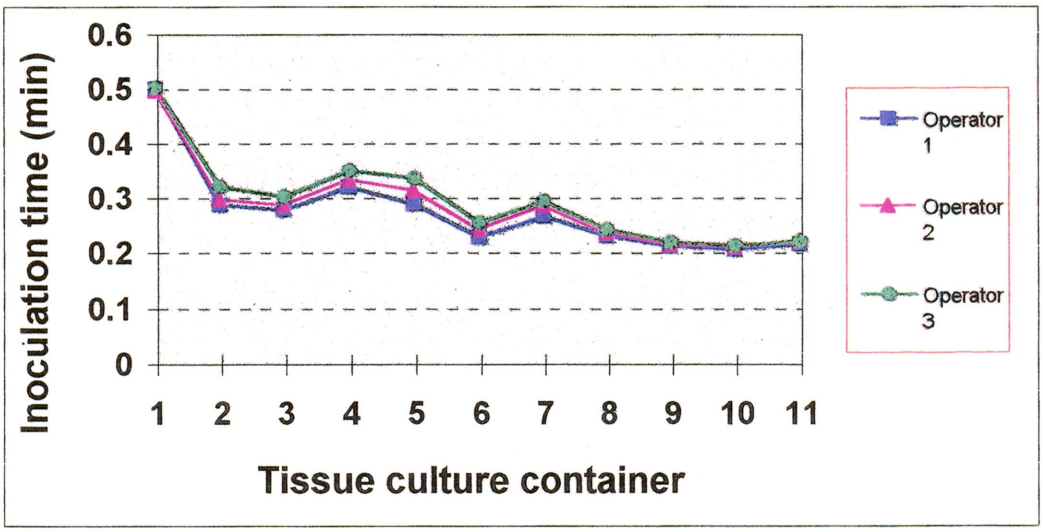

Figure 2.1 The influence of different tissue culture containers on the time of inoculation by different operators ($P < 0{,}001$). ($LSD_T = 0.096$) (Containers 1 to 11 are described in Table 2.1).

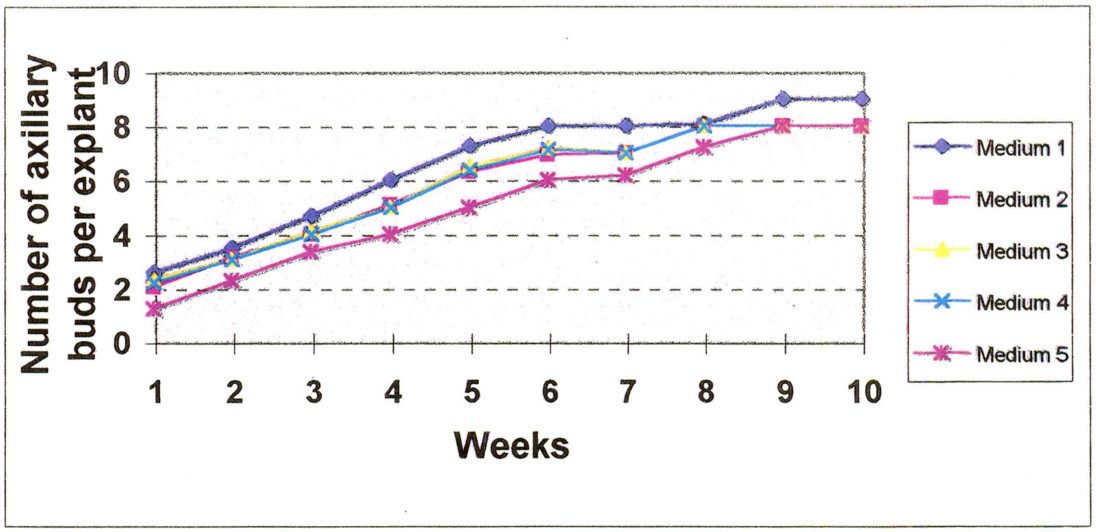

Figure 2.2 The influence of time and the effect of the medium on the productivity of axillary buds in potato tissue culture. (Medium 1 = MS supplemented with silver thiosulphate, Medium 2=Osborne, Medium 3=Regeneration medium, Medium 4= MS medium and Medium 5 = Philippine medium) ($P < 0{,}001$; $LSD_T = 0.267$)).

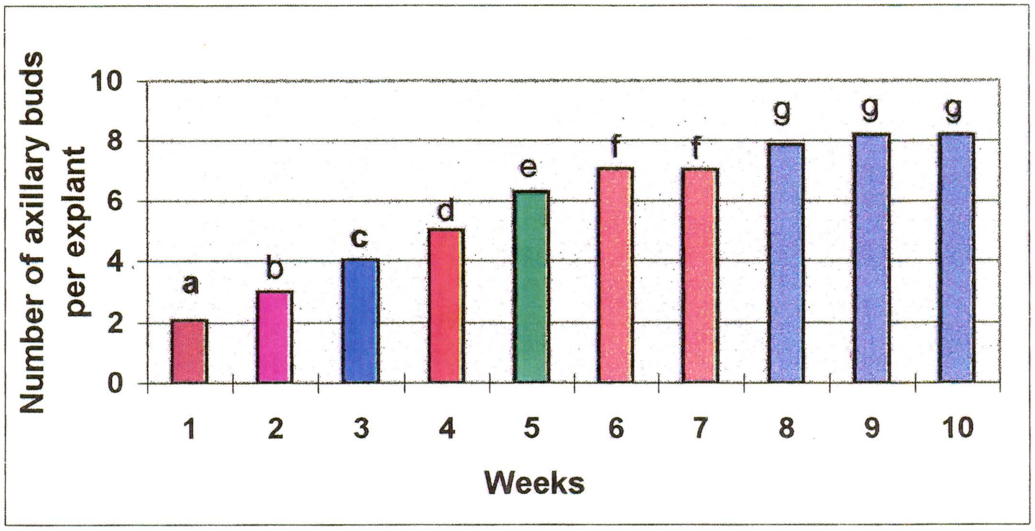

Figure 2.3 Total number of axillary buds produced by five potato cultivars in potato tissue culture during ten weeks on all media tested. Statistical analysis done by Tukeys multiple range test ($P< 0,001$, $LSD_T = 0,53$).

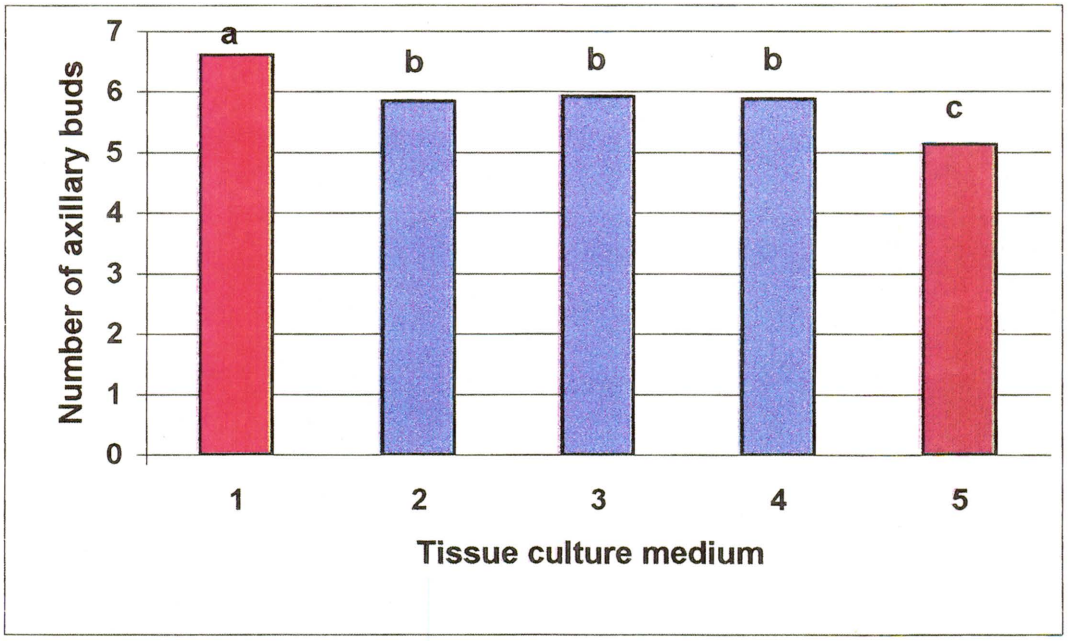

Figure 2.4 Number of axillary buds produced on five different tissue culture media of five potato cultivars after six weeks ($P < 0,001$, $LSD_T = 0,46$) (Medium 1 = MS supplemented with silver thiosulphate, Medium 2 = Osborne, Medium 3 = Regeneration medium, Medium 4 = MS medium and Medium 5 = Philippine medium) ($P < 0,001$). Differences due to medium and cultivar.

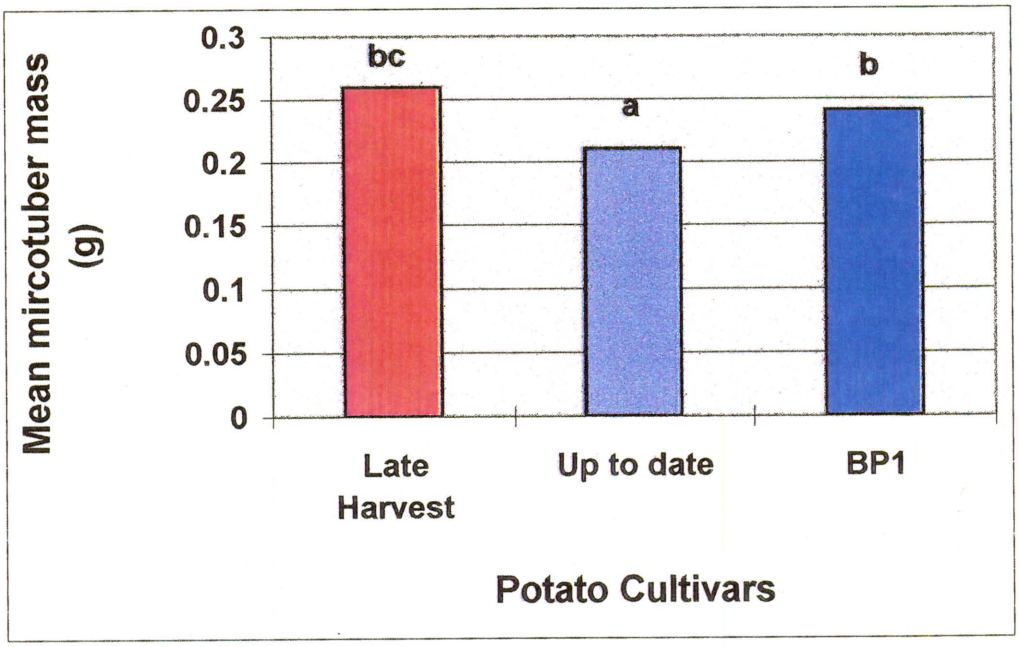

Figure 2.5 Mean microtuber mass of three different potato cultivars on chlorocholine chloride medium. ($P < 0{,}001$, $LSD_T = 0{,}027$).

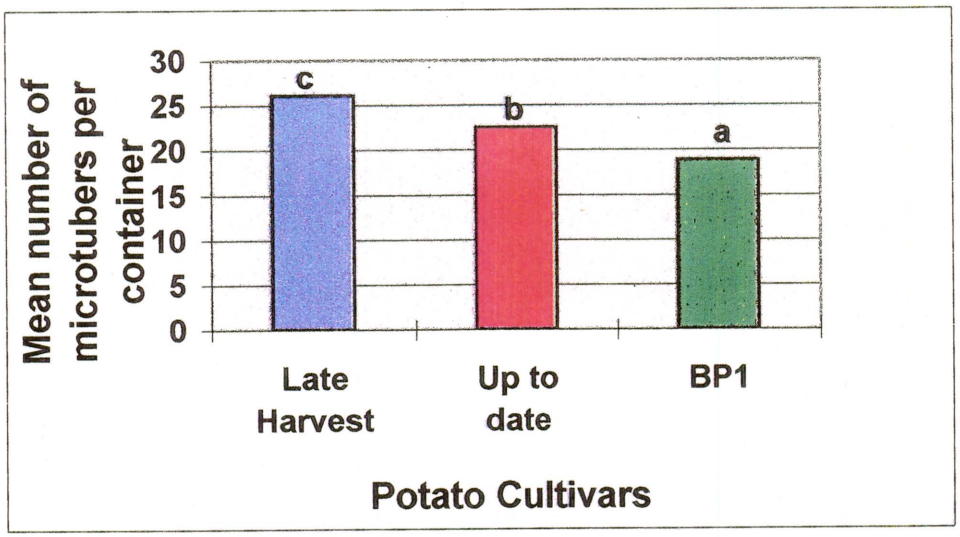

Figure 2.6 Mean number of microtubers produced by three different potato cultivars on chlorocholine choride medium. ($P < 0{,}001$, $LSD_T = 2.14$).

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3. OPTIMIZATION OF TECHNIQUES FOR *IN VITRO* CULTIVATION OF FIVE SOUTH AFRICAN POTATO CULTIVARS*

INTRODUCTION

There is a rapidly rising demand for food throughout the world. In South Africa hunger and malnutrition are found in many areas (Anonymous, 1992). Potatoes represent one of the main sources of food in many countries (Filho *et al.*, 1994), including South Africa. The South African potato industry is the biggest producer in Africa with a annual production of 1,4 million tonnes the past five years on approximately 58 000 hectares (Van Vuuren, 1997).

There is increasing awareness of the potential as well as limitations of tissue and cell culture techniques employed for the development of new lines with valuable characteristics for agriculture (Sabbah & Tal, 1990). Plant tissue culture has in recent years attracted growing interest from plant pathologists and breeders aiming for disease resistance, since it could meet the requirements for screening a large number of individuals in a small space (Chawla & Wenzel, 1987; Coleman *et al.*, 1991). It is important to develop an optimal *in vitro* system which can enable one to add selective agents to screen potato cultivars for better disease resistance. This is important since one has to ensure that clones which perform poorly *in vitro* do so because of the selective agent and not because growth in culture was not optimal. This study has not previously been done for South African potato cultivars. In order to develop an effective technique for the selection of potato somaclones or breeding lines with improved disease resistance, technology had to be developed to establish South African potato cultivars *in vitro*.

The present paper represents the first stage of a long-term project planned for the development of *Fusarium* resistant plants from tissue or cell culture. It deals with the development of optimal conditions for callus and cell suspention cultures for five South

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African potato cultivars.

MATERIALS AND METHODS

Plant material

In vitro derived leaves and stems (nodal explants) from virus-free potato plantlets of cultivars Late Harvest, BP1, Kimberley Choice, Up-to-Date and Vanderplank were used. The plantlets were maintained on MS medium (Murashige & Skoog, 1962) supplemented with 1.5 mg.dm^{-3} silver thiosulphate (STS). Cultures were incubated at 26 ± 1°C with a 16h photoperiod, at 55 µmol.m^{-2}.s^{-1}.

Preparation of callus

Potato leaves produced from four week old *in vitro* plantlets, approximately 1-1.5 cm in length, with the apical and basal 3 mm removed, were placed with the abaxial side in contact with the callus inducing medium. In addition stem segments produced from four week old *in vitro* plantlets, 10 mm in length, containing nodes but not leaves, were placed flat on nutrient media, consisting of modified MS salts and vitamins (Murashige & Skoog, 1962), 2.0 g.dm^{-3} casein hydrolysate, 30.0 g.dm^{-3} sucrose with varying concentrations of 2,4-dichloro-phenoxyacetic acid (2,4-D) and kinetin (KIN). Sixteen different concentration combinations of 2,4-D and KIN were used. KIN concentrations were 0.0, 0.25, 0.5 and 0.75 mg.dm^{-3} and 2,4-D concentrations 0.0, 1.0, 2.0 and 3.0 mg.dm^{-3}. Preliminary differential treatments with NAA and 2,4-D concentrations of 0.0, 1.0 3.0 and 5.0 mg.dm^{-3} and KIN concentrations 0.0, 0.1, 0.2 and 0.3 mg.dm^{-3} were done. These cultures were incubated at 26 ± 1°C and was done in the dark and in light. The pH was adjusted to 5.8 with 1M NaOH and 7.5 g.dm^{-3} agar (Biolab Agar Commercial gel, Merck) was used as the solidifying agent. Petri dishes (9cm diameter) were sealed with two layers of Parafilm and incubated in the dark at 26 ± 1°C for 20 days. Fifteen petri dishes were used per treatment, with 5 explants per petri dish.

Determination of callus growth

Determinations of fresh and dry mass are the parameters used most commonly for evaluating callus growth (Mottley & Keen, 1987). These methods are, however destructive and involve repeated handling of the calli. This leads to the disturbance of the contact between the calli and the media as well as the atmospheric composition in the container. To circumvent these constraints, a point-counting method, described by Mottley & Keen (1987) was used to evaluate callus growth. The area of the callus was estimated by counting points on a grid placed randomly over the base of an inverted petri dish. The points on the overlay are arranged in a matrix at 2 mm intervals. Only the points with their centres exactly on or inside the edge of the callus were counted. The area of the callus was calculated by using the following formula: $A = N \times D^2$, where A is the calculated area, N is the number of points counted and D the distance between the points.

Trial runs were conducted to determine the accuracy of greatest width determinations compared to point counts. Greatest width is another non-destructive method used by Mottley & Keen (1987), and is obtained by measuring the distance between the two furthest points on each callus surface with a ruler. Point counts as well as greatest width measurements and fresh mass (g) were done at 7 day intervals for 28 days.

Once the culture medium for optimal callus growth was selected, new callus was initiated on the selected medium from similar stem segments. After four weeks the explants had produced sufficient callus for it to be subcultured. The newly formed callus tissue was carefully dissected from the explant material and was transferred to fresh culture medium. The calli were subcultured every four weeks.

Preparation of cell suspension cultures

To initiate a cell suspension culture, 2.0 g (fresh mass) of friable callus was transferred to 100 cm^3 Erlenmeyer flasks containing 15.0 cm^3 liquid medium. The liquid medium was of the same composition as that used for optimal callus growth with 2.0 $mg.dm^{-3}$ 2,4-D,

0.25 mg.dm^{-3} KIN, 2.0 mg.dm^{-3} casein hydrolisate as well as 30.0 mg.dm^{-3} sucrose, but without agar.

The flasks were agitated continuously on a rotary shaker at 80 rpm under continuous dim light (10 μmol.m^{-2}s^{-1}) at 22 ± 1 °C. In order to establish a fine, fast growing suspension culture, the medium was replaced every seven days. On subculturing during the first three weeks in culture, the cells were allowed to settle to the bottom of the flask and the supernatant removed with a sterile pipette. Fresh medium was added and after the third week the suspension produced enough cells to be subcultured. With the first subculture into fresh medium large clumps were removed. Smaller clumps still occurred.

As the cell suspensions developed, they were transferred to 250 cm^3 Erlenmeyer flasks and later to 500 cm^3 Erlenmeyer flasks. The amount of media with cells was always 20 % of the volume of the flask, while 25 % of the suspension volume consisted of cells. Cell viabilities were determined by staining with FDA (Fluorescein diacetate) (Widholm, 1972; Larkin, 1976). The stained cells were inspected under a microscope with UV illumination to check for viability.

Determination of cell suspension growth

Growth curves were plotted over a period of 20 days using determinations of fresh mass, dry mass as well as packed cell volume (PCV) (Dixon, 1985, Dodds & Roberts, 1985). Fresh and dry mass determinations were performed every day for 21 days. Four replicates were used. A volume of 10 cm^3 of cell suspension was filtered through 7.0 cm ashless nr.41 Whatman filter paper by means of a Buchner funnel and a vacuum pump. After determining the fresh mass of the cells, plus filter paper, they were dried in an oven at 80 °C for 48h. The dried filter paper with cells were allowed to cool in a dessicator, before dry mass was determined. The wet and dry mass of the filter paper were measured separately and subtracted to obtain the fresh and dry mass of callus.

To determine PCV, a known volume of cell suspension was transferred to a tapered 15 cm^3 graduated centrifuge tube, and centrifuged for 5 min at 180 x g in a bench centrifuge, the volume of the pellet measured and PCV determined by the formula PCV = (Vol pellet/ Vol suspension) x 100 % (Dixon, 1985).

RESULTS AND DISCUSSION

Preliminary differential treatments with NAA and 2,4-D gave a good indication of the cultivars' reaction on medium containing NAA. Results indicated that tissues cultured under light were unable to produce either roots or shoots. According to the results media containing 2,4-D produced significantly more callus than media containing NAA ($P< 0,005$). (Results not shown).

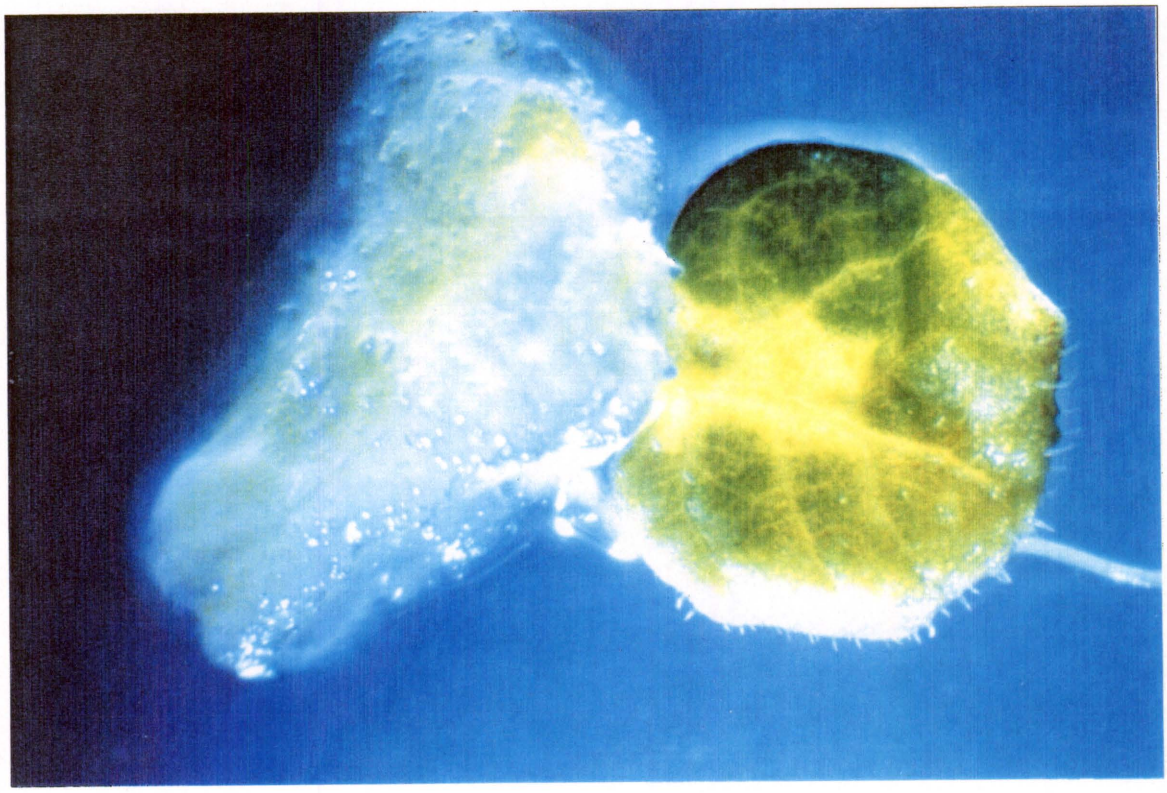


Figure 3.6: Nodal explant tissue produced callus before the leaf tissue.

It was noticed that the stem tissue produced callus before the leaves (Figure 3.6). This was in accordance with results obtained by Quraishi *et al.*, (1987). In this study stem callus was more friable than the callus produced by the leaves. The medium that gave optimal callus growth (most) as well as the best callus colour and texture (for production of cell suspension cultures) for all the cultivars, was the modified MS with 2mg.dm^{-3} 2,4-D and 0.25mg.dm^{-3} KIN (Figure 3.1). These calli were found to be white and friable (Figure 3.7).

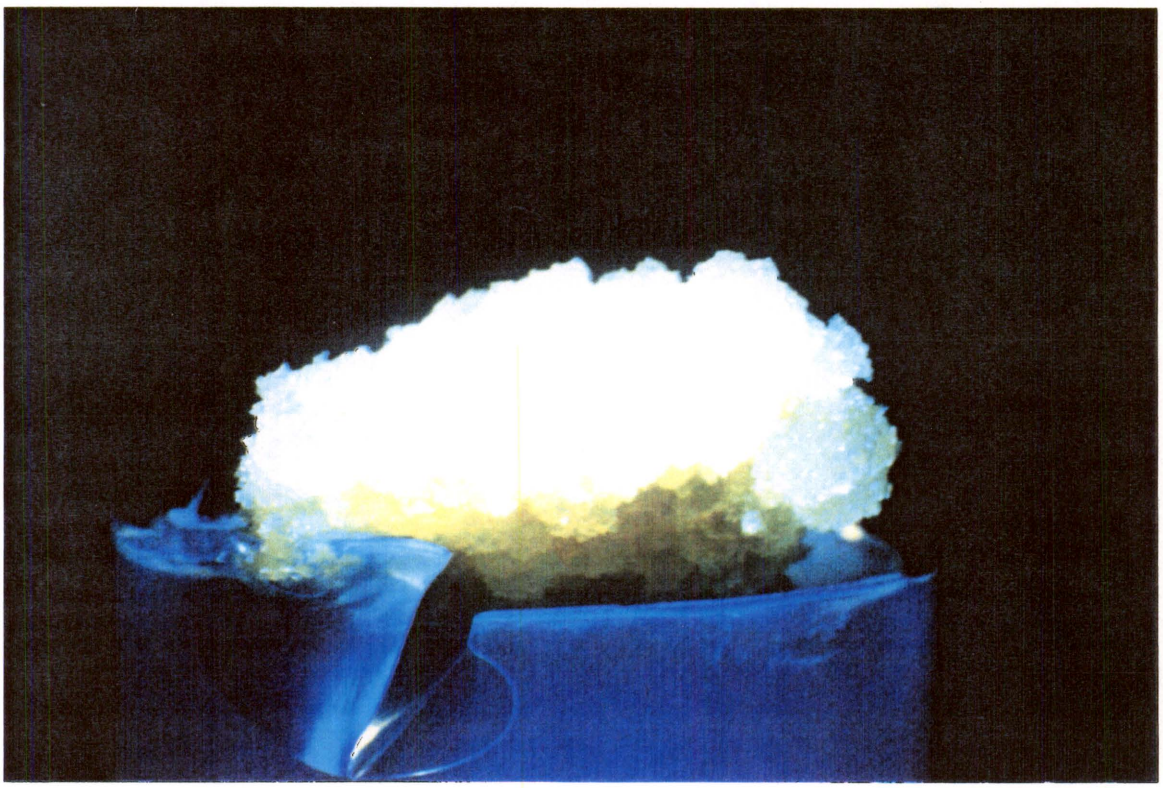


Figure 3.7: White friable callus produced on MS medium supplemented with 2 mg.dm^{-3} 2,4-D and 0.25 mg.dm^{-3} KIN

Callus grown on media containing NAA turned brown. Root formation was also observed in cultures grown on media with NAA whereas no roots were formed on media with 2,4-D. Murashige (1973) noted that auxins not only induced cell division, but also stimulated the formation of roots. The development of roots was undesirable as friable, white callus was required for initiating cell cultures. The treatments without 2,4-D and NAA formed no callus and the axillary buds started to grow which indicated that auxins were necessary for callus growth. This was in accordance with results obtained by Okazawa *et al.*, (1967) who also noted that exogenously applied auxin is important for initiating callus growth.

For the differential treatment (Table 3.1), used in this study, NAA was omitted from the experiment, because of the brown callus and roots that had developed in preliminary trials.

Table 3.1 Differential treatment with 2,4-D and Kinetin induce callus formation and growth.

Kinetin ($mg.dm^{-3}$) \ 2,4-D ($mg.dm^{-3}$)	0.0	1.0	2.0	3.0
0.0	1	2	3	4
0.25	5	6	7	8
0.5	9	10	11	12
0.75	13	14	15	16

Results suggest that the potato cultivars required 2,4-D and KIN to induce callus formation and growth. 2,4-D represses organised development and shoot regeneration, and promotes cell enlargement (Gavinlertvatana & Li, 1980). This requirement has been observed in several other plant species (Murashige *et al.*, 1974; Pierik & Steegmans, 1975; Gavinlertvatana & Li, 1980).

Greater width determinations of callus gave similar results to the point-count method and consequently only the latter was used. The results obtained by calculating the areas of the callus grown on the media as shown in the differential treatment are shown in Figure 3.1 and fresh mass Figure 3.2. The data in Figures 3.1 and 3.2 shows that the area and mass respectively of the callus of all the cultivars increased with an increase in 2,4-D concentration up to 2.0 $mg.dm^{-3}$. At higher 2,4-D concentration (3.0 $mg.dm^{-3}$) the area

of the callus was slightly less than at 2.0 mg.dm^{-3} 2,4-D. In the literature the range of 2,4-D concentrations used for induction of callus in potato varied between 2.0 mg.dm^{-3} and 3.0 mg.dm $^{-3}$ (Bajaj & Dionne, 1967; Lam, 1977; Sharp & Larsen, 1979; Gavinlertvatana & Li, 1980 and Wareh *et al.*, 1989;). Chapman (1955) used 7.0 mg.dm^{-3} 2,4-D, but no shoots were regenerated thereafter. This study is in agreement with others in the literature.

Media containing 0.25 mg.dm^{-3} KIN and 2.0 mg.dm^{-3} 2,4-D (treatment 10) gave significantly better results than any of the other treatment media for all the cultivars (P <0,001) (Figure 3.1). It was also apparent that the presence of KIN increased the callus area significantly. Palni *et al.*, (1988) indicated that cytokinin stability is inversely related to auxin concentration. It is important to have the right combination of supplements in the callus culture medium in order to control the friability of the callus tissue in cell suspension cultures (Bajaj & Dionne, 1967; Hulme, *et al.*, 1992). The age of the callus culture plays a major role in dissociation of the callus (Bajaj & Dionne, 1967). Tobacco calli required only a short period of gentle agitation in liquid medium (Vasil & Hildebrandt, 1965), whereas potato calli needed more time to produce a good suspension (Bajaj & Dionne, 1967). The cultivar Late Harvest produced significantly more callus at 2.0 mg.dm^{-3} 2,4-D and 0.25 mg.dm^{-3} KIN than all the other cultivars. There was no statistical difference between 'Kimberley Choice', 'Up-to-Date' and 'BP1', whereas 'Vanderplank' produced significantly less callus than the other cultivars (Figure 3.1 and 3.2).

The techniques for the *in vitro* cultivation of many plants is genotype specific. The most important factor in the successful establishment and maintainence of *in vitro* cultures has been shown to be the genotype. Genotypes respond differently to different levels of nutrients and growth regulators in the medium (Ulrich & Mackinney, 1969).

The callus area at 0.5 mg.dm^{-3} KIN was less than the callus area at 0.25 mg.dm^{-3} KIN, indicating that KIN becomes inhibitory at higher levels (Figure 3.1). Sharp & Larsen (1979) noted that a high concentration of auxin and a low concentration of cytokinin are required for callus induction. Results indicated that the effect of kinetin was optimal at 0.25mg.dm^{-3}. When the KIN concentration exceeded 0.5mg.dm^{-3}, the area of the callus

was reduced.

Results showed that KIN was necessary for callus growth, since the maximum area obtained without any KIN was less than the area obtained on the medium containing the maximum concentration of KIN (Figure 3.1). This confirmed that there was a correlation between the auxin and the cytokinin concentration. The growth of the callus depended on the presence of both auxin and cytokinin. Modified MS salts and vitamins, containing 2.0mg.dm^{-3} 2,4-D and 0.25mg.dm^{-3} KIN was subsequently used to subculture the callus in order to produce enough callus for the initiation of a cell suspension culture.

The cultivar 'Late Harvest' produced significantly more callus than the other cultivars ($P < 0,001$) and 'Vanderplank' significantly less callus than the other cultivars ($P < 0,001$) whereas no significant differences was observed between the remaining three cultivars (Figure 3.1 & 3.2). Despite producing the least, 'Vanderplank' still produced enough friable white callus for growth. It was therefore decided that this medium would be used in future with all other cultivars and only if insufficient callus growth is obtained, other auxins and/or cytokinins will be tested.

Dessication and discolouration was noticed in four week old cultues. This was in agreement with the findings of other researchers (Dodds & Roberts, 1985). These calli were therefore subcultured every four weeks.

Dissociation of the callus in cell suspension cultures depended on it's friability. The callus of the potato cultivars Late Harvest, Vanderplank, Kimberley Choice, Up-to-Date and BP1 required two months before a good cell suspension was established (Figure 3.8).

Figure 3.8: Cell suspension culture of cultivar Late Harvest after two months. (Left: Before subculturing; Right: After subculturing)

Figures 3.3, 3.4 and 3.5 show growth curves, as determined by fresh mass, dry mass and PCV. All three figures demonstrate sigmoidal growth. Cell suspension cultures required regular subculture at more frequent intervals than callus cultures. In order to maintain the viability of the culture, it must be subcultured during the exponential phase (Dodds & Roberts, 1985). In order to keep the cell suspension of the potato cultivars viable and growing at its maximum potential, the suspension must be subcultured during the exponential phase between day five and 11 when it is in an active state of growth. Subsequently, the cell suspensions were subcultured every seven days.

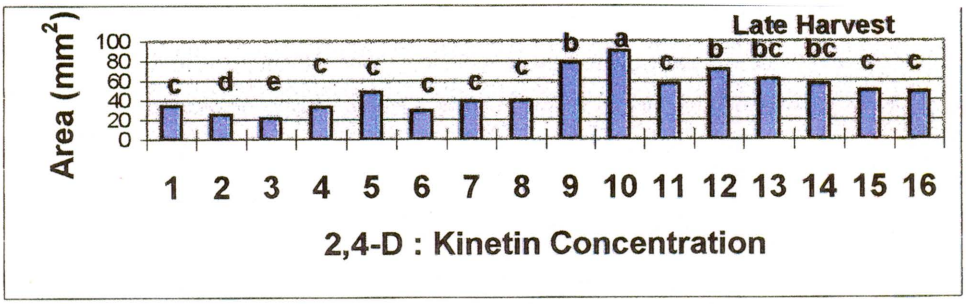
The growth curve as determined by measurements of dry mass, fresh mass and PCV (Fig. 3.3, 3.4 and 3.5) over a period of 21 days, reached a maximum growth rate on day eleven, except for 'Vanderplank' which reached a maximum on day 13, indicating that cell division was terminated on these days. Between days 11 and 14 (14 and 17 for 'Vanderplank') there was a stationary phase. The suspension was actively growing between day five and 10 (day six and 13 for 'Vanderplank') after which there was a decrease in mass. After 14 days there was a decrease in growth.

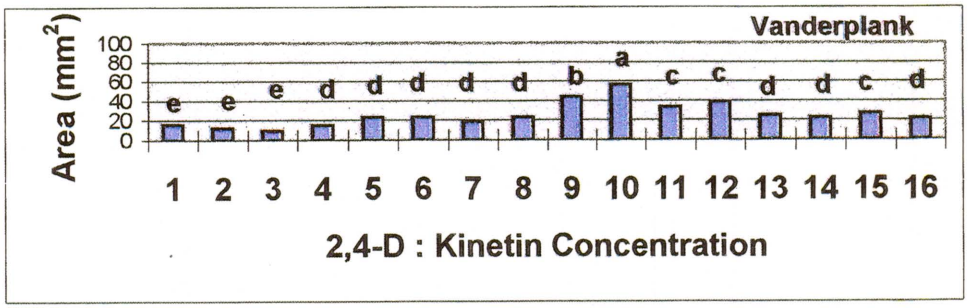
When the PCV was determined, approximately 35 % of the volume of a cell suspension consisted of nutrient medium. After the cell suspension was subcultured, more than 75% of the volume of the suspension consisted of medium. There was no significant difference in the time to reach the maximum growth between cultivars 'Late Harvest', 'Kimberley Choice', 'Up-to-Date' and 'BP1', but 'Vanderplank' took significantly longer to reach maximum growth than all the other cultivars ($P < 0,001$) (Figure 3.5). Although this was the case, 'Vanderplank' still produced enough cells in suspension on day seven to be able to subculture it on that day.

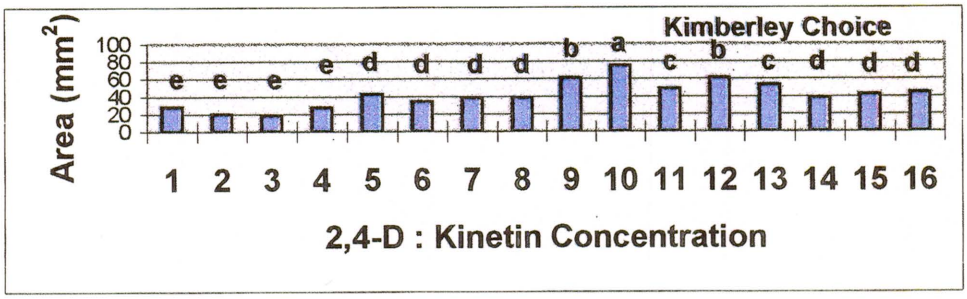
'Late Harvest' produced significantly more cells at day 11 (maximum growth) ($P < 0,001$) than the other cultivars, but there was no significant difference between the other cultivars.

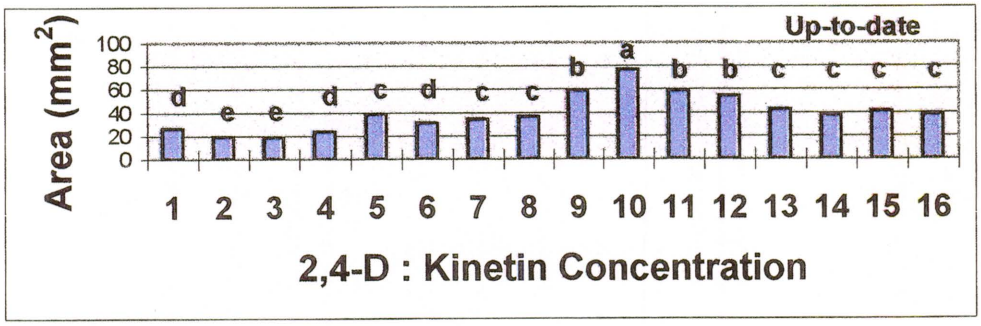
CONCLUSION

Results obtained indicated that the three techniques used to determine cell suspension growth gave similar results (Figures 3.3, 3.4 and 3.5). Of the three techniques determination of PCV proved to be the least time consuming and most economical. It is recommended that this method be used in further studies. Results obtained indicates that this cell culture approach is effective in obtaining optimal callus and cell suspension cultures for South African potato cultivars. Results indicated that the best medium for induction of callus was MS supplemented with 2,0 $mg.dm^{-3}$ 2,4-D and 0,25 $mg.dm^{-3}$ kinetin.









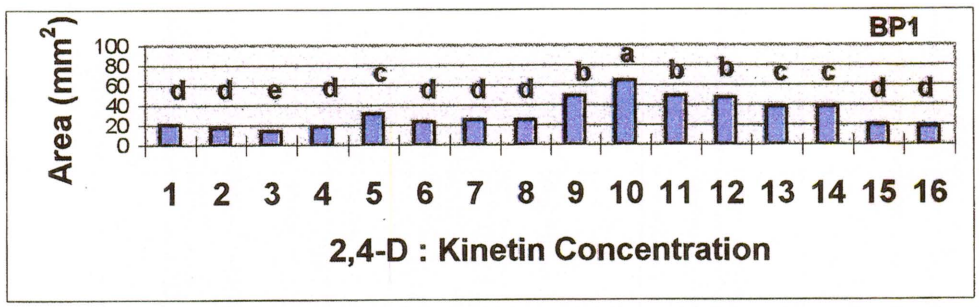


Figure 3.1 Area (mm²) of callus of five potato cultivars grown at different Kinetin and 2,4 -D concentrations (LSD_T = 5,67). Treatments are explained in Table 3.1.

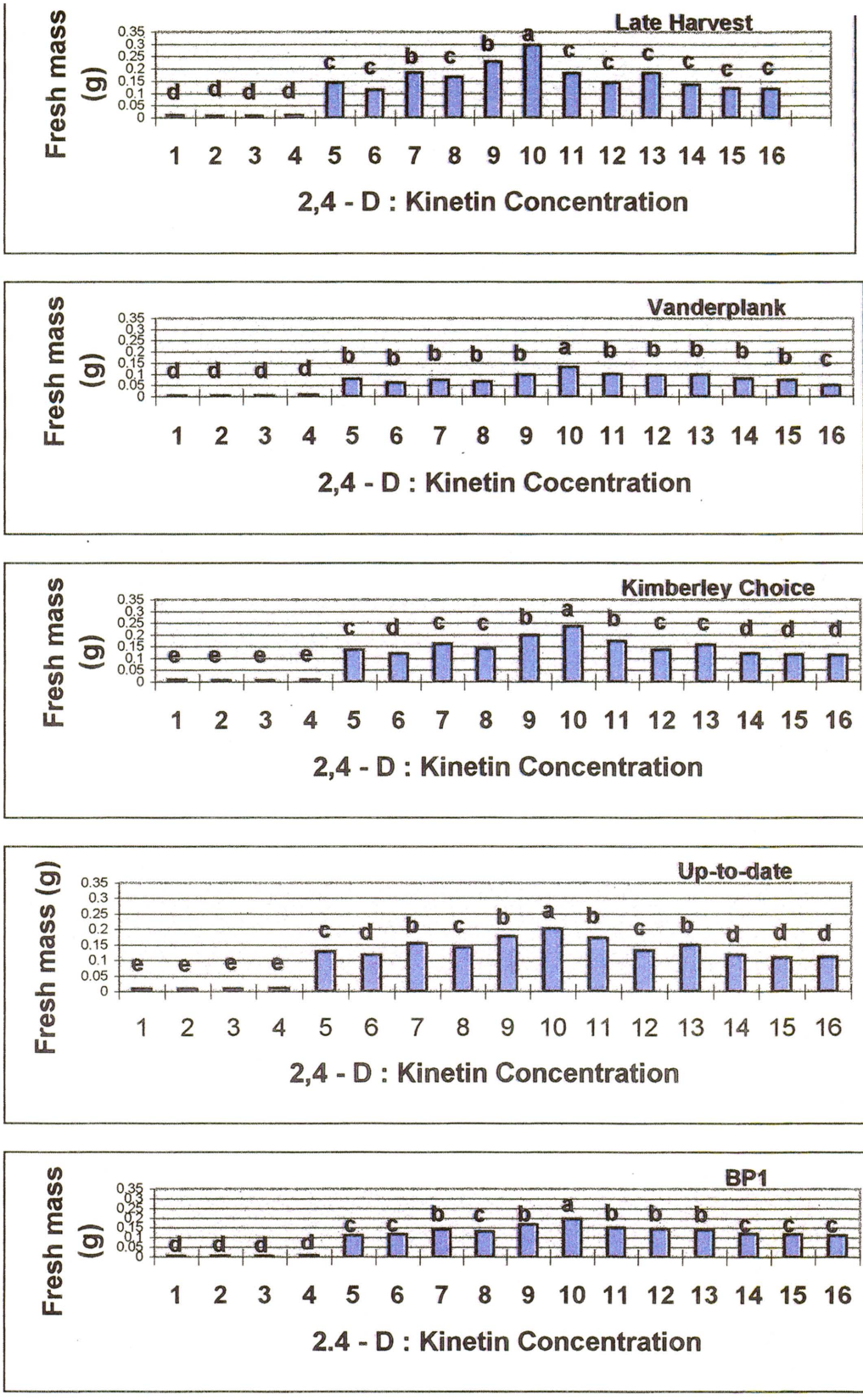


Figure 3.2 Fresh mass (g) of callus of five potato cultivars grown at different KIN and 2,4 -D concentrations (LSD_T = 0,036). Treatments are explained in Table 3.1.

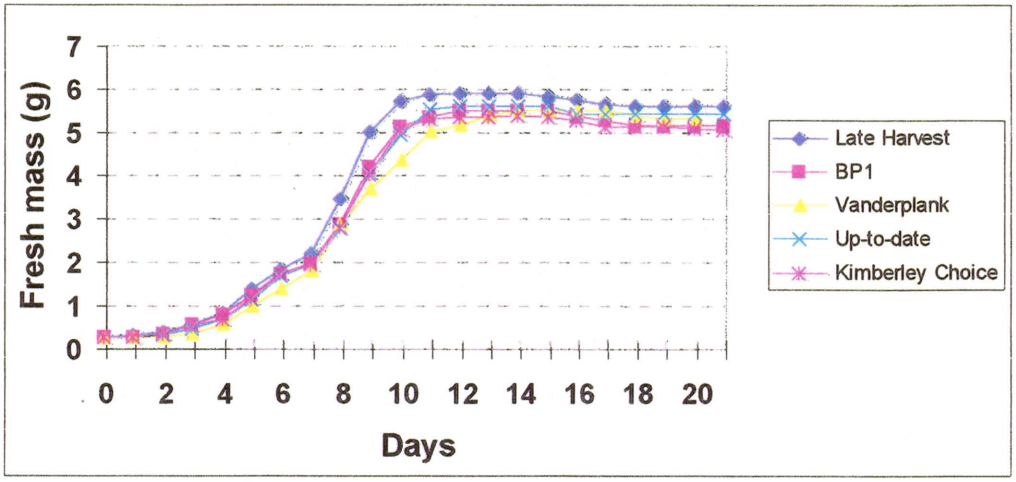


Figure 3.3 Growth curve of 5 potato cultivars as plotted by using determinations of fresh mass (g) (LSD_T = 0,097).

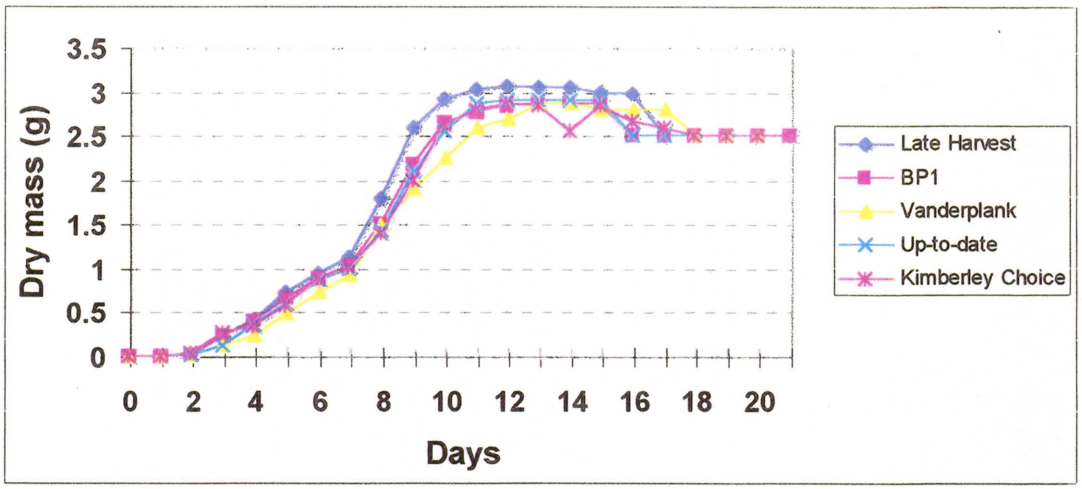


Figure 3.4 Growth curve of 5 potato cultivars as plotted by using determinations of dry mass. (LSD_T = 0,033).

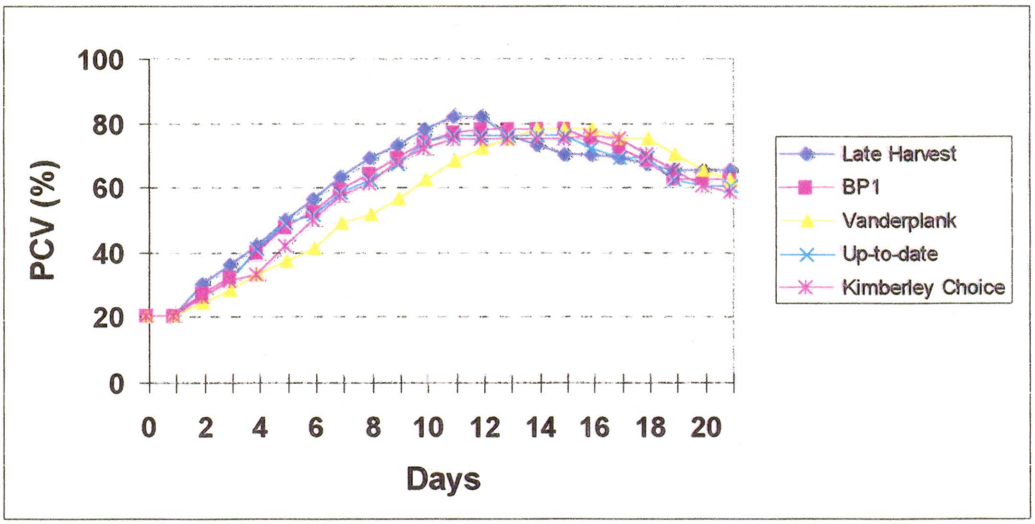


Figure 3.5 Growth curve of 5 potato cultivars using determinations of packed cell volume (PCV) (LSD_T = 1.19).

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4. REGENERATION OF PLANTLETS FROM CELL CULTURES OF FIVE POTATO CULTIVARS OF SOUTH AFRICA*

INTRODUCTION

Potato represents one of the main sources of food in many countries. (Filho *et al.*, 1994). In South Africa potato is one of the major horticultural crops and yet it's production faces several problems due to diseases, pests and weeds. Efficient *in vitro* regeneration procedures are important for the exploitation of *in vitro* selection technology in potato improvement.

Asexually reproduced organisms, such as commercial potato cultivars, do not normally reproduce sexually and therefore genetic variation is limited (Coleman *et al.*, 1990). *In vitro* methods may overcome this restriction. Determining the conditions that enhance shoot regeneration for various genotypes would increase the practicability of this approach. High regeneration rates would increase the chances of acquiring shoots with the desired resistance.

Regeneration of potato plants is influenced by various factors including the physical environment, culture medium, genotype and type of explant (Jacobson, 1981; Fish & Jones, 1988; Coleman *et al.*, 1990; M'Ribu & Veilleux, 1990). The most important determinant of regeneration is the genotype of the plant and this can inhibit the exploitation of *in vitro* techniques.

The objective of this study was to optimize the conditions and nutrient requirements for the regeneration of plantlets from cell suspension cultures of five potato cultivars of South Africa.

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*Venter, S.L., Thiart, S., Ferreira, D.I. & Eicker, A.Submitted to the Journal of the Southern African Society for Horticultural Sciences.

MATERIALS AND METHODS

Maintenance of suspension cultures

Cell suspension cultures as described in chapter three of five potato cultivars, 'Late Harvest', 'BP1', 'Up-to-Date', 'Vanderplank' and 'Kimberly Choice', were used.

Cell suspension cultures were subcultured every seven days in liquid MS medium (Murashige & Skoog, 1962) supplemented with 2mg.dm $^{-3}$ 2,4-D and 0,25 mg.dm^{-3} kinetin.

Determination of plating density

To initiate plant regeneration, the cells must be plated at a specific density to obtain optimal growth. The cells had to be counted prior to plating. A new technique for the counting of cells was developed. Due to large cell sizes and the presence of aggregates a small chamber was developed to count the cells under a microscope. The amount of cells per microscope field was counted. In order to calculate the number of cells in 1 cm^3, the conversion factor for the microscope as well as the dilutions were taken into account and this factor for 10 x magnification was 688,37 and for 20 x magnification 2 598,75.

In an attempt to break the aggregates, an experiment was conducted using an 8 % (w/v) aqueous chromium trioxide (CrO_3) solution. One cubic centimeter of packed cells that was diluted in a 1:9 ratio, was added to 2 cm^3 of 8 % (w/v) CrO_3. The cells were heated for 30 min in a waterbath at 70°C and then agitated in a Vortex for 10 min. to break the aggregates. Heat treatments of 10, 15, 20, 25 and 30 min with a Vortex time of 5 minutes and 10 min were evaluated.

On day seven, the cell suspensions were centrifuged for five min at 200 x g in a bench centrifuge in sterile centrifuge tubes.

To determine the optimum plating density, cells were plated at the following dilutions: 2x, 6x, 10x, 100x and 1000x where x = 7,51 x 10^4. Two cubic centimeters of the diluted suspensions were plated per petri dish (qcm diameter). Visual determination of the best dilution was performed after two weeks of incubation (Figure 4.1). The dillution of which the individual microcalli could be seen was the best.

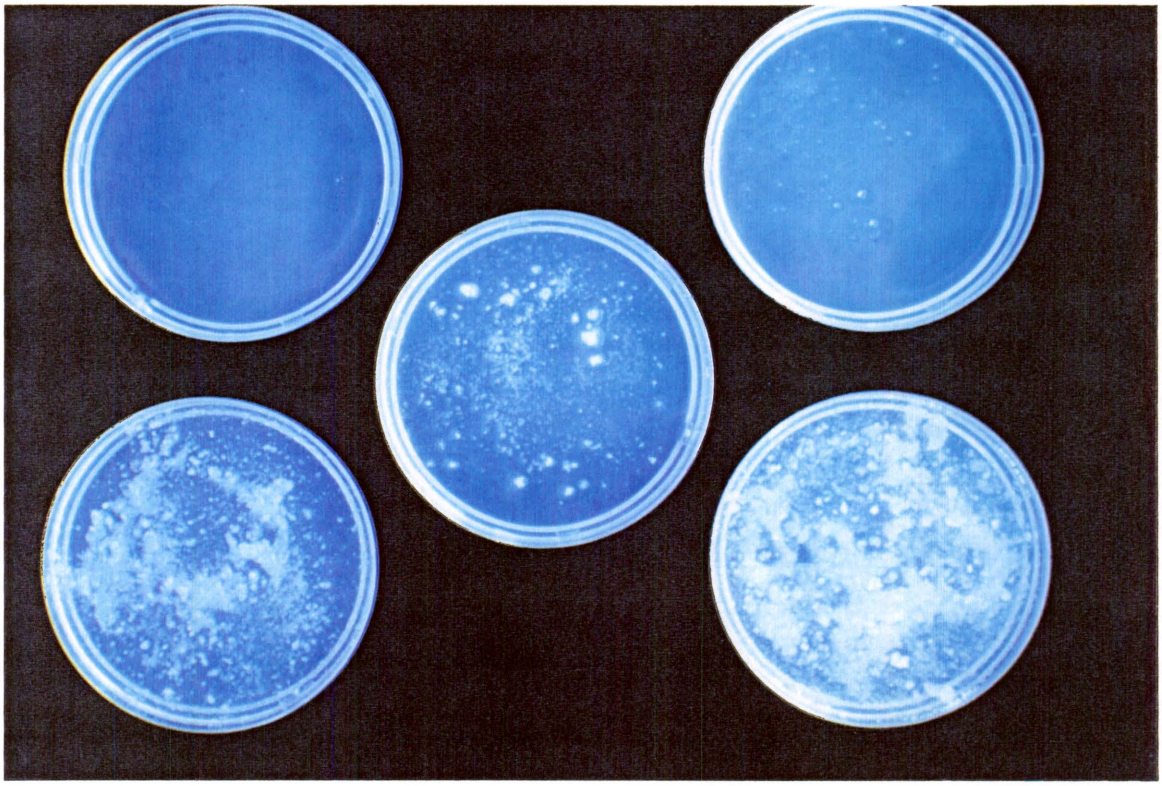

Figure 4.1: Optimal plating density (individual micro calli could be distinguished) of cells was the 10x dilution (x=7,51 x 10^4) (middle). (Top left: 1 000 x, Top right: 100 x, bottom left: 6x bottom right 2x) . With the 1 000 x and 100x no micro calli was produced with the 2x and 6x individual micro calli could not be distinguished).

After determining the optimum plating density, the packed cells were diluted with media in a 1:9 ratio. Two cubic centimetres of suspension were plated on 20 cm^3 of medium consisting of basic MS, supplemented with 2 $mg.dm^{-3}$ 2,4-D, 0.25 $mg.dm^{-3}$ KIN, 2 $mg.dm^{-3}$ casein hydrolysate, 30 $g.dm^{-3}$ sucrose with 8 $g.dm^{-3}$ agar (Biolab Agar Commercial Gel Merck) in sterile petri dishes. The pH was adjusted to 5.8 with 1M NaOH.

The petri dishes with plated cells were incubated at 26±1 °C and a 16h photoperiod under cool white fluorescent tubes (55 $\mu mol.m^{-2}s^{-1}$). The petri dishes were sealed with two layers of Parafilm to avoid contamination. One day after plating, the superfluous liquid was removed by means of sterile pasteur pipettes. The petri dishes were sealed again with two layers of Parafilm. When the calli reached the size of 2-3 mm in diameter, they were subsequently transferred to medium for callus greening, described by Cassels et al.,(1986) & Cassels *et al.*, (1987).

Twenty five calli were placed in every petri dish on 20 cm^3 of callus greening medium. Twenty dishes per treatment were used.

Regeneration of shoots

The calli greened within two weeks at 26±1 °C with a 16h photoperiod under cool white fluorescent tubes (55$\mu mol.m^{-2}s^{-1}$). After two more weeks on the greening medium, the calli were transferred to three different treatments for shoot regeneration, as well as to the shoot regeneration medium as described by Lam (1977).

The different treatments for plant regeneration compared various concentrations of IAA to zeatin, NAA to BAP and NAA to zeatin. The basis of the media for these treatments consisted of MS salts and vitamins with 0.5 $mg.dm^{-3}$ GA_3.

Five enlarged and greened calli per petri dish were placed on 20 cm^3 of medium in petridishes (9 cm^3 in diameter). The petri dishes were sealed with two layers of Parafilm

and incubated at 26± 1°C with a 16h photoperiod under cool white fluorescent tubes (55 $\mu mol^{-2}s^{-1}$).

The shoots were carefully dissected from the callus tissue and regenerated shoots were placed on modified MS salts and vitamins, containing 0.01 $mg.dm^{-3}$ NAA, 0.1 $mg.dm^{-3}$ GA_3, 25 $g.dm^{-3}$ sucrose, 7 $g.dm^{-3}$ agar (Biolab Agar Commercial Gel Merck) with the pH adjusted to 5.5 in plastic tubs (250 cm^3). The shoots rooted within two weeks. These *in vitro* plantlets were transferred to soil and were grown in a greenhouse.

RESULTS AND DISCUSSION

Plating density

The aggregates made it difficult to count the cells and to calculate the volume of suspension to be used. Cells could be counted after a 30 min heat treatment in an aqueous CrO_3 solution followed by 10 min Vortex. At heat treatments of 20-30 minutes with a Vortex time less than 10 min, aggregates were still a problem, but cells could be counted allthough not accurately. Heat treatment less than 20 min had no effect and cells could not be counted.

Cell aggregation in plant suspension cultures is a confounding factor in the performance of growth experiments and interpretation of growth data (Kubek and Shuler, 1978). Cell aggregates complicate the rapid measurement of cell mass, volume and number. The number of cells per cubic centimeter of packed cells was *circa* 3,8241x10^6 for ;Late Harvest', *circa* 3,8061x10^6 for 'BP1', *circa* 3,7967x10^6 for 'Kimberley Choice', ±3,7863x10^6 for 'Up-to-Date' and *circa* ±3,7629x10^6 for 'Vanderplank'.

After two weeks in culture, it was clear that the 10x (circa 7,531 x 10^5 $cells.cm^{-3}$) dilution was the optimal density for the cells to be plated (Figure 4.1). Individual calli could not be distinguished at 2x and 6x dilutions, whereas the 100x and 1000x dilutions were too dilute. It was important for the individual calli to be distinguished in order to be certain

of their single cell origin.

The number of cells plated per petri dish was *circa* $7{,}531 \times 10^5$ (10x dilution) for all cultivars. Within two weeks the cells enlarged to microcalli (2-3mm in diameter). The microcalli were removed weekly and transferred to callus greening media as described by Cassels *et al.*, (1986, 1987). Within two weeks the calli greened and enlarged to 5 mm in diameter. These calli were transferred to the different treatments and the shoot induction medium described by Lam (1977).

Shoot regeneration

After 6 months none of the cultivars developed shoots on the different treatments with different concentrations of IAA and zeatin, NAA and zeatin and NAA and BAP.

The shoot and root formation are controlled by the ratio of cytokinin and auxin in the medium. Low auxin and high cytokinin concentrations in the medium result in the induction of shoot morphogenesis (Sharp & Larsen, 1979).

The first shoots developed from the callus on the medium for shoot induction described by Lam (1977) within 32 days (Figure 4.2). The regeneration rate of shoots was very low. Of 5 000 microcalli of 'Late Harvest', only 458 plantlets were recovered (9,16%) and from 5 000 microcalli of 'Kimberley Choice', only 288 plantlets developed (5,76%), 219 from 'Up-to-Date' (4,38%), 326 plantlets from "BP1" (6,52%) and from 'Vanderplank' 137 plantlets (2,74%). In two subsequent experiments these percentages were more or less the same. Low frequency of shoot initiation from cell suspensions may be due to loss of morphogenic ability. This can happen when these cultures are older than 12 months and have been maintained by subcultures. Jacobsen (1987), however, found that in some dihaploids, plants could be regenerated in calli older than two years. It was found that 2,4-D represses organised development and shoot regeneration (Gavinlertvatana & Li, 1980). The longer the culture is exposed to 2,4-D, the longer it will take to regenerate plants. It may also be possible that the genotype does not respond

well to regeneration. Theoretically it should be possible to regenerate plants. Some genotypes are, however, more responsive than others (Meredith, 1979). Murashige (1973) suggested that a relative high concentration of auxin combined with a low concentration of cytokinin will initiate roots, whereas the reverse will lead to shoot formation. From results obtained it is obvious that large numbers of microcalli should be produced to obtain enough plants to be evaluated. It is possible that the difference in the response of the potato cultivars tested came from genotype x medium interactions and could be prevailed by alteration of medium compositions. This was found to be the case for barley (Hanzel, *et al.*, 1985). The physiological status of tissue culture may also play an important role (Duncan *et al.*, 1985).

CONCLUSION

The *in vitro* requirements for the regeneration of plantlets from cell cultures of the five potato cultivars of South Africa have been established and although the regeneration potential of the cultures is relatively low, the value of the established techniques are of importance. A new technique for the counting of potato cells in cell suspension cultures was developed and optimum plating density for production of micro calli developed. It may be possible that higher cytokinin concentration will lead to enhanced shoot formation. This should be confirmed by more extensive research.

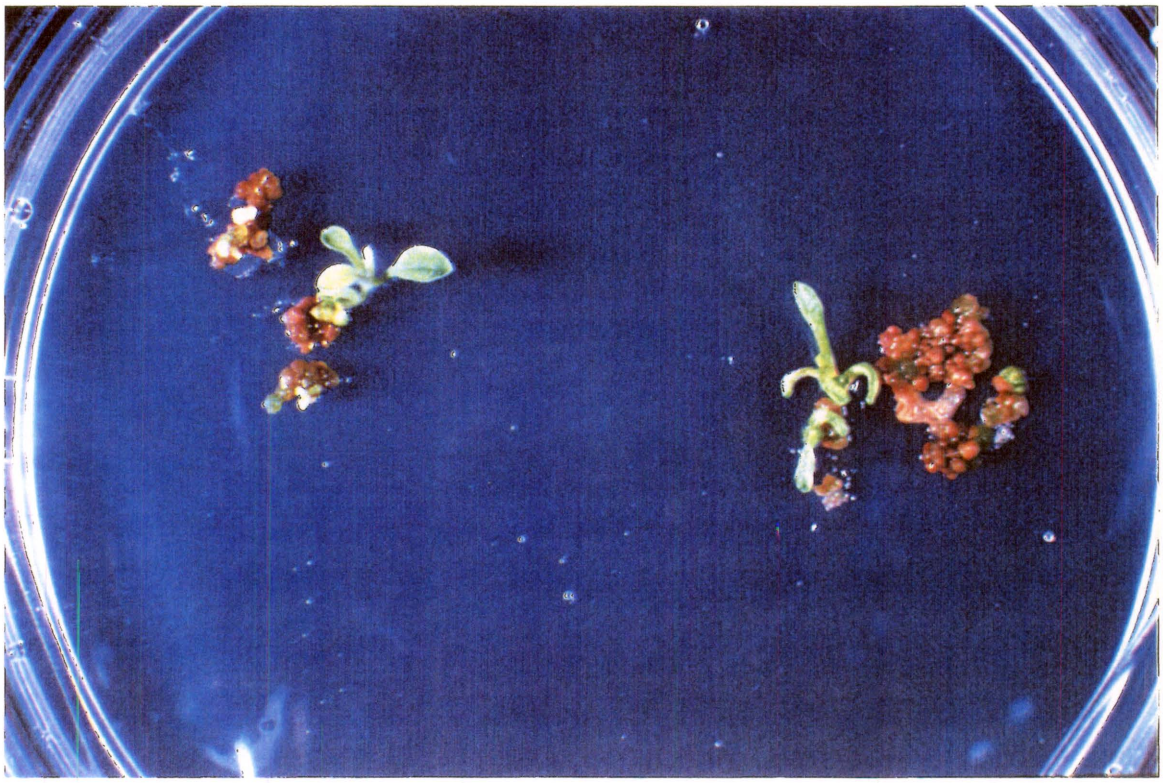

Figure 4.2: Shoots developed from Late Harvest callus on shoot induction medium.

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5. OPTIMIZATION OF PLANT REGENERATION FROM POTATO LEAF DISCS*

INTRODUCTION

Efficient *in vitro* regeneration procedures are a pre-requisite for the exploitation of *in vitro* selection technology in potato improvement. Regeneration of potato plants is influenced by various factors including the physical environment, culture medium, genotype and type of explant (Jacobson, 1981 & 1987; Fish & Jones, 1988; Coleman *et al,*. 1990; M'Ribu & Veilleux, 1990). The most important determinant of regeneration is the genotype of the plant and this can inhibit the exploitation of *in vitro* techniques. Foulger & Jones (1986) found that standard regeneration protocols could be modified slightly and result in improved performance of the more recalcitrant genotypes, while Fish & Jones (1988) demonstrated considerable difference in tissue responsiveness between related potato genotypes.

Determining the conditions that enhance shoot regeneration for various genotypes would increase the practicability of this approach. High regeneration rates would increase the chances of acquiring shoots with the desired resistance. A major disadvantage of using leaf explants is the occurrence of somaclonal variation because of the callus phase (Visser, 1991). This can be minnimised by keeping the callus phase as short as possible. Regeneration from leaf explants can be divided into three phases : the initiation of callus, the initiation of shoots on this callus and shoot development. The published procedures for potato regeneration from leaf discs can be classified into one-step methods where a single culture medium is used for all these phases (Tavazza *et al.*, 1988; Keil *et al.*, 1989), two-step methods (Wheeler *et al.*, 1985; Fish & Jones, 1988; Wenzler *et al.*, 1989) where callus initiation takes place on one medium and shoot formation and development on another, and three-step methods where different media are used for each of the three phases (Hulme *et al.*, 1992).

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*Venter, S.L., Thiart, S., Ferreira, D.I. & Eicker, A. Submitted to the South African Journal of Science

The objective of this study was to optimize the conditions and nutrient requirements for the regeneration of plantlets from leaf discs of five South African potato cultivars.

MATERIALS AND METHODS

In vitro derived leaves from virus-free potato plantlets from cultivars 'Late Harvest', 'BP1', 'Kimberley Choice', 'Up-to-Date' and 'Vanderplank' were used for regeneration experiments. The plantlets were maintained on MS medium (Murashige & Skoog, 1962) containing 1.5 $mg.dm^{-3}$ silver thiosulphate (STS). Incorporation of STS into the tissue culture media increased the surface area of the *in vitro* leaves (Venter *et al.*, 1997*)* All cultures were placed at 26±1 °C with a 16h photoperiod, at 55 $\mu mol\text{-}m.^{-2}s^{-1}$.

The leaf disc regeneration medium consisted of MS medium supplemented with 20 $g.dm^{-3}$ sucrose, 2 $mg.dm^{-3}$ zeatin (added after autoclaving), 0.02 $mg.dm^{-3}$ 1-naphtyl acetic acid (NAA), 0.02 $mg.dm^{-3}$ gibberellic acid (GA_3), 1.5 $mg.dm^{-3}$ STS and 7.5 $g.dm^{-3}$ agar (Biolab Agar, Commercial Gel, Merck) with pH 5,8. Potato leaves produced from 4 - 6 week old *in vitro* plantlets approximately 1 - 1.5 cm in length, with the apical and basal 3 mm removed, were placed with the adaxial side facing upwards on plates containing the regeneration medium. Leaf discs were subcultured onto fresh medium once a week and when callus production could be seen on the cut surfaces of the leaves (5 - 8 weeks depending on the cultivar), the leaves were transferred to regeneration medium without auxin to stimulate shoot production. Preliminary trials proved that is was neccessary to transfer leaf discs to fresh medium once a week. Emerged shoots (1 cm in length at least) were rooted on modified MS medium with 0.1 $mg.dm^{-3}$ GA_3, 0.01 $mg.dm^{-3}$ NAA and 25.0 $g.dm^{-3}$ sucrose. The number of explants exhibiting shoot regeneration, the number of shoots per explant and the total number of shoots per cultivar were determined. To determine the effect of the type of explant on regeneration, five leaf disc pieces (as described above) and stem internodes (3-5 mm length) were cultured on regeneration medium. The number of explants exhibiting regeneration and the number of shoots per explant were determined after 14 weeks on shoot regeneration medium.

The effect of subculture interval on regeneration were determined. Leaf discs were subcultured weekly, once every two weeks and once a month.

RESULTS AND DISCUSSION

Cultivar differences (genotypic variation) were observed for regeneration frequency and number of shoots per explant (Table 5.1). Shoot regeneration was generally high among all the cultivars tested (Figure 5.1). 'Late Harvest', 'Up-to-Date' and 'BP1' formed shoots on most explants (70-85 %) with many shoots per explant and 'Kimberley Choice' and 'Vanderplank' formed shoots on most of the explants (65 %) but with few shoots per explant (Table 5.1). 'Late Harvest' and 'Up-to-Date' produced significantly more shoots per explant than 'Kimberley Choice' and 'Vanderplank' (Table 5.1). 'Late Harvest' also produced significantly more plants in total (469) than any of the other cultivars, after 15 weeks 'Up-to-Date' produced significantly more regenerated plants (342) than 'BP1', 'Kimberley Choice' and 'Vanderplank'. There were no statistical difference between 'Kimberley Choice' and 'Vanderplank'. 'BP1' produced a total of 307 plants after 15 weeks, 'Kimberley Choice' a total of 220 and 'Vanderplank', 217. 'Late Harvest', 'Up-to-Date' and 'BP1' formed shoots over an extended period (week 4 - week 15) whereas 'Kimberley Choice' and 'Vanderplank' formed shoots only during a certain period (week 7 - week 11) Fish & Jones (1988) & M'Ribu & Veilleux (1990) also observed variation for shoot regeneration period.

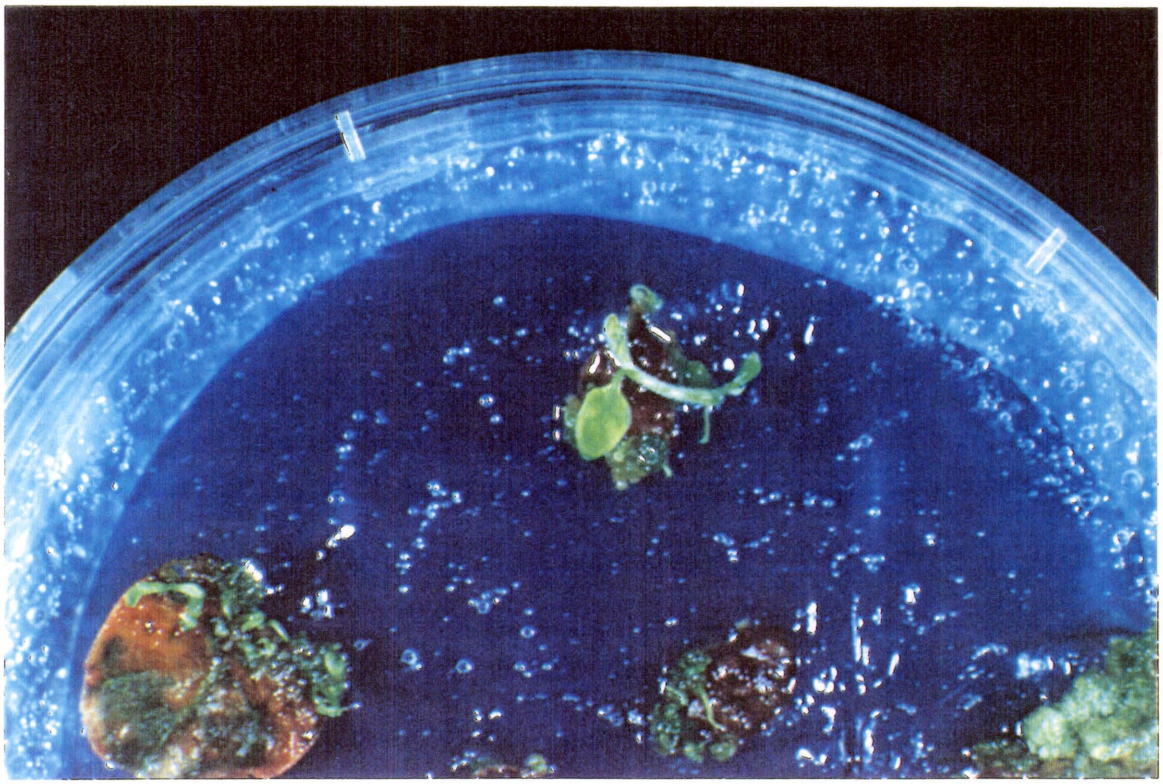

Figure 5.1: Regenerated shoots produced from Late Harvest leaf discs after 36 days in culture on shoot regeneration medium consisting of MS medium supplemented with 20g.dm^{-3} sucrose, 2 mg.dm^{-3} zeatin, 0.02 mg.dm^{-3} 1-naphtyl-acetic acid (NAA), 0.02 mg.dm^{-3} GA_3, 1.5 mg.dm^{-3} STS and 7.5 mg.dm^{-3} agar. (Biolab Agar commercial Gel, Merck)

Table 5.1 Shoot regeneration from leaf discs after 15 weeks for five potato cultivars

Cultivar	Mean number of explants	Mean number of days before shoot formation	Regeneration frequency		Mean shoot number per explant
			Mean number of explant producing shoots per treatment	% of explants producing shoots per treatment	
'Late Harvest'	50	28[a]	43	86	9,8[a]
'Up-to-Date'	50	30[a]	38	76	8,3[a]
'BP1'	50	49[b]	35	70	6,9[b]
'Kimberley Choice'	50	41[c]	38	76	5,8[c]
'Vanderplank'	50	52[c]	38	76	5,7[c]
LSD_T (5 %)	-	8	-	22	3

Significant differences were observed in regeneration frequency (number of explants producing shoots) among the cultivars (Table 5.1), but although the shoot number per explant was variable all the cultivars in this study regenerated at high frequencies. The number of days before shoot formation and the number of regenerated shoots per explant varied significantly among the cultivars and were negatively correlated ($r = -0,69$, $P < 0.001$). Thus cultures that formed shoots early also tended to have more shoots. This was also found by M'Ribu & Veilleux (1990) for *Solanum phureja* monoploids.

Table 5.2 **Shoot regeneration after 15 weeks for explants that were subcultured at various intervals, for five potato cultivars**

Weeks between subculture	Number of explants [a]	Mean number of days before shoot formation	Regeneration frequency		Mean shoot number per explant
			Mean number of explants producing shoots per treatment	% of explants producing shoots per treatment	
1	100	27	93	93	5,9[a]
2	100	33	84	84	5,5[a]
3	100	36	42	42	3,2[b]
4	100	48	26	26	1,7[c]
LSD_T (5%)		6,8	17	17	4,2

[a] Twenty explants per cultivar for five cultivras (20 x 5 = 100)

Leaf discs subcultured to new regeneration medium once a week formed shoots earlier and had higher regeneration frequencies than those subcultured once every four weeks (Table 5.2). Explants subcultured once every two weeks were intermediate and did not differ significantly from those subcultured once a week, but differed significantly from those subcultured once every three or four weeks (Table 5.2).

Table 5.3 **Shoot regeneration after 15 weeks for leaf and stem explants of five potato cultivars**

Type of explant	Number of explants [a]	Mean number of days before shoot formation	Regeneration frequency		Mean shoot number per explant
			Mean number of explants producing shoots per treatment	% of explants producing shoots per treatment	
Leaf	100	27[a]	91	91	5,9[a]
Stem	100	32[b]	74	74	2,1[b]
LSD_T (5%)		5	20	20	2,8

[a] Twenty explants per cultivar for five cultivars (5 x 20 = 100)

Leaf explants exhibited higher regeneration frequencies than stems (Table 5.3). There were significant differences in the number of days before shoot formation as well as the number of shoot produced per explant on the two types of explants (Table 5.3). Shoot regeneration from leaf, stem and tuber explants has previously been reported in several potato cultivars (Wheeler *et al.*, 1985; Ochatt & Caso, 1986). M'ribu & Villeux (1990) stated that because of morphological differences the explants do not represent equivalent tissues, therefore direct comparison of explants may not be appropriate. It is important however that various tissues can be used as explants for shoot formation. The five South African potato cultivars vary in the relative number and size of stems and leaves, therefore the more abundant tissue (leaves) can be used. This confirms the report of M'Ribu & Villeux (1990).

CONCLUSION

South African potato cultivars varied for earliness of shoot formation, shoot regeneration frequency, number of shoots formed and pattern and duration of shoot formation in tissue culture. There was no direct relationship between regeneration frequency and number of shoots per explant. What is important however was that the regeneration frequency for

all the cultivars was high and that this method can successfully be used for regeneration of potato plantlets from leaf discs for South African cultivars.

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6. PRODUCTION OF FUSARIC ACID BY *FUSARIUM OXYSPORUM*

INTRODUCTION

Plant pathogens produce various kinds of toxic compounds to culture media as well as in plant tissues (Drysdale, 1982). These compounds cause a series of morphological and biochemical changes in plant tissues and contribute to the pathogenicity or virulence of the organisms producing the toxin. *Fusarium* sp. are known to produce several toxins including fusaric acid (Drysdale, 1982; Hardborne, 1983). This toxin has been studied extensively (Julien, 1988; Kern, 1972; Löffler & Morris, 1992; Matsui & Watanabe, 1988; Mégnégneau & Branchard, 1988). The production of fusaric acid by a bulb rot producing *Fusarium* sp. was first reported by Löffler & Morris (1992). This paper is the first report of the production of fusaric acid by *Fusarium oxysporum* Schlecht, emend Snyd. & Hans, which causes dry rot of potato tubers. A correlation was found between virulence of isolates and fusaric acid production. It was found that Fusaric acid production may play a major role in the development of dry rot in potato tubers (Chapter 7).

MATERIALS AND METHODS

Fungal isolates. Three isolates of *Fusarium oxysporum* (F.o.1, F.o.4 and F.o.6) which had been isolated from potato tubers with dry rot symptoms, were used. Their virulence had been verified by Theron & Holz (1989).

Culture filtrate. Erlenmeyer flasks containing 30 mℓ culture medium were inoculated with single agar plugs (1 mm^3) of one of the three isolates. The following culture medium was used: 0.74 $g.dm^{-3}$ $MgSO_4 \cdot 7H_2O$; 0.045 $g.dm^{-3}$ $MnSO_4 \cdot 4H_2O$; 0.018 $g.dm^{-3}$ $ZnSO_4 \cdot 7H_2O$; 5,0 $\mu g.dm^{-3}$ $CuSO_4 \cdot SH_2O$; 0.084$g.dm^{-3}$ $FeSO_4 \cdot 7H_2$); 0.11 $g.dm^{-3}$ Na_2EDTA; 0.1 $g.dm^{-3}$ myo-inositol; 2.0 mg glycine; 0.5 $mg.dm^{-3}$ nicotinic acid; 0.5 $mg.dm^{-3}$ pyridoxine.HCl; 0.1 $mg.dm^{-3}$ thiamine.HCl; 10.0 g glucose; 2.0 $g.dm^{-3}$ L-asparagine; 1.0 $g.dm^{-3}$ KH_2PO_4;

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*Venter, S.L., Steyn, P.J. & Steyn, H.S.F. This chapter was published in Potato Research 39 (1996) 79-84.

5.0 $\mu g.dm^{-3}$ biotin; 1 000 mℓ distilled water. The flasks were incubated at 22±1°C on an orbital shaker in the dark (50 r.p.m.) for the duration of the experiment.

Mycelial growth. Mycelial growth was determined every 24 h for 30 days. The mycelium and culture medium from five flasks were individually separated by a Buchner funnel, the mycelium weighed, and the mean fresh mass (g) recorded.

Toxin preparation and extraction. Crude filtrates and a control of culture medium only were acidified with 1N HCl and extracted with an equal volume of ethyl acetate. The organic phase was removed and dried under vacuum, the residues dissolved with 5 mℓ methanol and stored at -20 °C. The fusaric acid peak (HPLC) of the samples was identified by co-elution with pure fusaric acid added to the sample. Extraction efficiency was determined by spiking half of the sample with 0.015 mg $m\ell^{-1}$ pure fusaric acid (5-butyl picolinic acid) (Sigma Chemical Company, U.S.A.) prior to acidification. Eight replicates were extracted.

Analysis of fusaric acid. Culture filtrates were collected every 24 h for thirty-two days and tested for the presence of fusaric acid using reversed phase High Performance Liquid Chromatography (HPLC) on a Bondclone 10 C18 column (10 μm, 300 x 3.9 mm). A 50 $\mu\ell$ aliquot of culture filtrate, culture medium or pure fusaric acid was injected and eluted isocratically with eluting buffer. The buffer was prepared by mixing (v/v) 40% methanol and 60% of an aqueous solution of 0.62mM Na_2EDTA and 2% H_3PO_4 (Julien, 1988). The absorption was recorded with a UV detector at 254 nm, using a Beckman system Gold model injector, pump and UV detector Model 168. Standard curves were established with pure fusaric acid. The column was stored in 100% methanol and equilibrated with eluting buffer 1 h before use. Samples were injected as methanol solutions.

RESULTS AND DISCUSSION

Extraction efficiency. Fusaric acid eluted in a reproducible manner as a sharp peak with an elution time of 5.56 min and a temperature of 20±1°C. Pure fusaric acid co-injected with the sample increased the height of the peak, and the product eluted with this peak had the

characteristic UV spectrum of fusaric acid. The extraction efficiency (% fusaric acid recovered) was determined from day 0 to day 4 for all three isolates by spiking half of the sample with 0.015 mg/mℓ pure fusaric acid. Fusaric acid (85.46%) was recovered with a coefficient of variance of 8,1%.

The growth- and fusaric acid production curves for the three isolates are shown in Fig. 6.1A-C. The variation of 92.0 %, 89.7% and 93.0% encountered for the fresh mass (Fig 6.1 A-C) can be described by quadratic curves, and the variation of 99. 8%, 99.8% and 98.5% encountered for fusaric acid production (Fig 6.1 A-C) can be described by exponential curves. Isolate F.o.1 showed the least mycelium growth and isolate F.o.6 the most; isolate F.o.1 produced the most fusaric acid and F.o.6 the least. Both these differences were significant ($p<0.001$). The growth curves for all three isolates showed a logarithmic growth phase from days 2 -18, after which a short stationary phase followed. Mycelium growth decreased after 20 days. At day 20 the fresh weight reached 1.46 g for isolate F.o.6, 1.26 g for isolate F.o.4 and 1.14 g for isolate F.o.1.

Mycelial growth and fusaric acid production (Table 6.1)

Most variation resulted from the main factor, which was days (Table 6.1). The variation explained by this factor was 96.1%, leaving 3.9% for the isolates. With reference to total variation, 88.3% of the variance was explained by days, with only 3.6% by isolates. The interaction of days and isolates explained 4.0% of the total variance. The variation between days and isolates and the interaction of days and isolates was statistically significant ($p<0.001$).

Table 6.1 : Analysis of variance of the results obtained from the growth (g fresh weight) measured over 32 days of three isolates of *Fusarium oxysporum* with five replicates.

Source of variation	Degrees of freedom	Sum of squares	Mean of squares	F-value	p-value
Factors	23	45.231634			
F. oxysporum isolates	2	1.754980	0.77490	114.73	<0.001
Days	21	43.476654	2.070317	270.70	<0.001
Interaction	306	3.991474			
F. oxysporum- days	42	1.972383	0,046961	6.14	<0.001
Residual	264	2.019099	0,007648		
Total	329	49.223118			
CV = 9,7%					

In Table 6.2 it is apparent that most variation (99.6%) again occurred as a result of days against 0.37% for isolates. Of total variation, 99.2% of the variance was explained by days, while the interaction days x isolates explained 0.55%. The variation between days and isolates and the interaction of days and isolates was statistically significant ($p<0.001$). The interaction according to mycelial growth (4.0%) was more important than that according to fusaric acid production (0.04%), but in both cases it was low.

Table 6.2 : Analysis of variance of the results obtained from fusaric acid production (mg $m\ell^{-1}$) measured over 32 days by three *Fusarium oxysporum* isolates with eight replicates

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value	p-value
Factors	20	0.126573			
F. oxysporum isolates	2	0.000473	0.000237	114.73	<0.001
Days	18	0.126	0.007000	270.70	<0.001
Interaction	425	0.000572			
F. oxysporum- days	36	0.000699	0,0000194	6.14	<0.001
Residual	389	0.000508	0,000000131		
Total	445	0.127			
CV = 1,8%					

The fusaric acid production curves showed a lag phase from days 0 - 5 and an exponential growth phase from days 6 - 18. After day 18 a stationary phase was reached and the fusaric acid concentration remained constant from then until day 32, when the experiment ended. The concentration of fusaric acid differed significantly between the different isolates. On day 20 the difference was 0.042 mg $m\ell^{-1}$ for isolate F.o.1, 0.040 mg m ℓ^{1} for F.o.4 and 0.038 mg ml^{1} for isolate F.o.6. When determining the fusaric acid production it is important to determine the concentration after the stationary phase has been reached.

CONCLUSION

It is apparent that mycelial growth cannot be used to measure or estimate fusaric acid production, because the functions explaining them differ. Growth curves from specific isolates could be used to determine the concentration of fusaric acid in culture filtrate after specific lengths of time.

ACKNOWLEDGEMENT

We wish to thank Niël Theron for supplying us with the *F. oxysporum* isolates, Marlene Pienaar for helping with the HPLC analyses and Annatjie Msiza for assistance with the extractions.

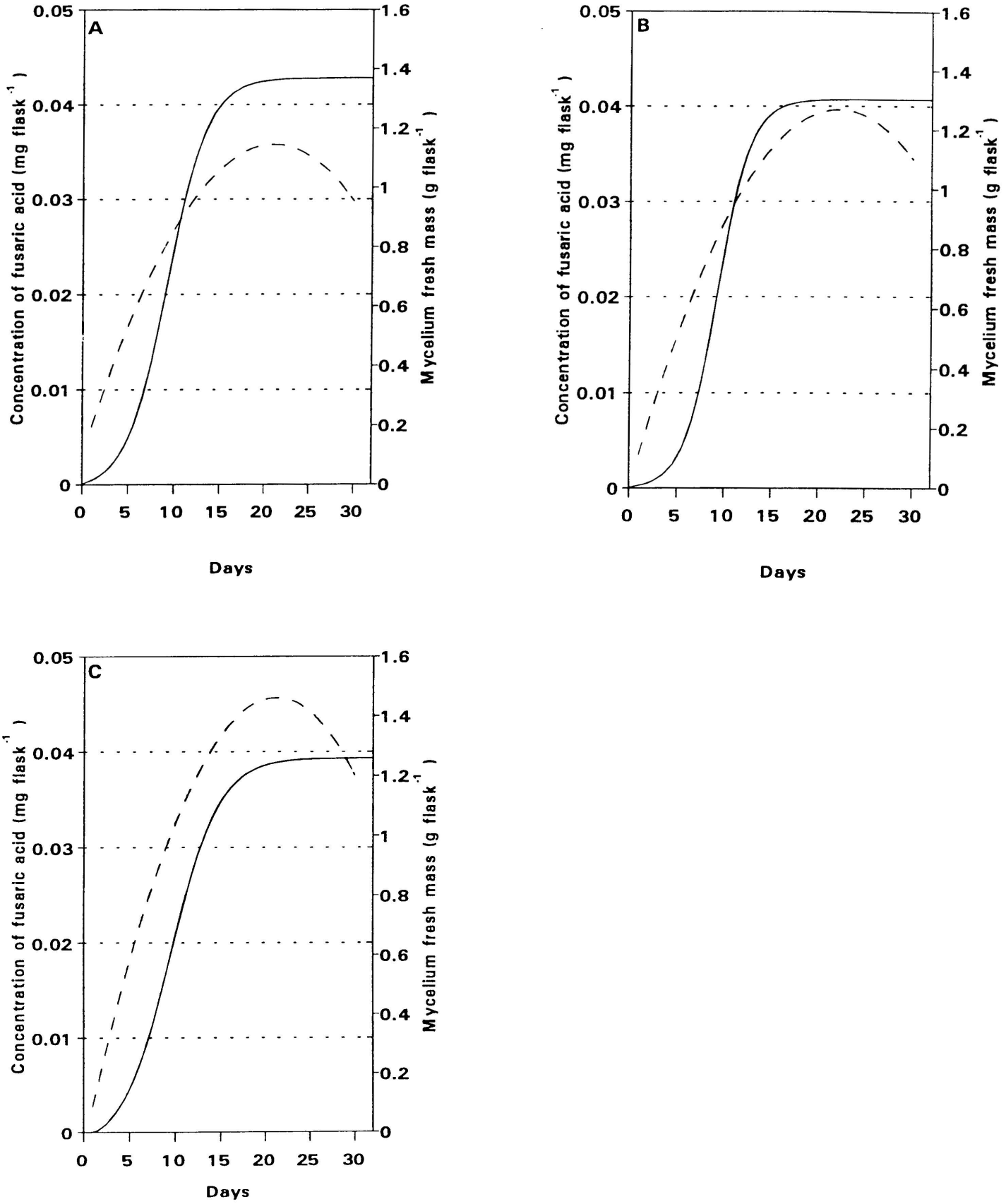

Figure 6.1 : Fusaric acid production (-) and mycelium growth (---) of isolates of *Fusarium oxysporum*. A = isolate F.0.1, B = F.o.4, C = F.o.6.

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7. CORRELATION BETWEEN FUSARIC ACID PRODUCTION AND VIRULENCE OF ISOLATES OF *FUSARIUM OXYSPORUM* THAT CAUSES POTATO DRY ROT IN SOUTH AFRICA*

INTRODUCTION

Fusarium oxysporum Schlect. Emend Snyd. & Hans. causes dry rot, stem-end rot and wilt of potatoes (*Solanum tuberosum L*) (Nelson *et al.*, 1981). Fusarium dry rot is mainly a post harvest disease and can become a major problem when infected potatoes are stored. Breeding for potato cultivars resistant to *Fusarium* species causing dry rot is an essential control strategy world-wide. Genetic modification of plants for disease resistance requires resistant individuals to be identified among large populations of susceptible plants. This is usually done by inoculating with the pathogen (Yoder 1983). Several authors have suggested the use of toxins produced by pathogens, for identification of disease resistant plants *in vitro* or *in vivo* (Gracen *et al.*, 1971; Galston 1974; Earle 1978; Wenzel 1985; Daub 1986; Branchard 1984; Brettel & Ingram 1979). Before a toxin can be used reliably as a surrogate for a pathogen it must convincingly be proven to play a causal role in disease development and if the toxin is pathologically important, the role in disease development should be defined.

Phytotoxins may either be a pathogenicity- or a virulence factor (Yoder 1980; Yoder 1983; Toyoda *et al.*, 1991). A pathogenicity factor is required by the producing pathogen to cause any disease at all, whereas a virulence factor is not necessary to initiate disease but, if present, changes the degree of disease development (Yoder 1980). Fusaric acid is produced by many *Fusarium oxysporum* formae sp. which cause diseases of many important crops (Mégnégneau & Branchard 1988). The possible involvement of fusaric acid, a non-specific toxin, in disease development has been reviewed by Pegg (1981). The aim of this study was to determine the role of fusaric acid in the development of potato dry rot caused by *Fusarium oxysporum.*

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MATERIALS AND METHODS

Fungal isolates. Twelve isolates of *Fusarium oxysporum* which had been isolated from potato tubers with dry rot symptoms were used. Their virulence had been verified by Theron & Holz (1989). The isolates differed in their ability to cause disease symptoms (virulence).

Virulence tests. Conidial suspensions of the twelve isolates were prepared as described by Venter *et al.*, (1992). Unblemished potato tubers of cultivars BP1, Kimberley Choice and Late Harvest were disinfected for 15 min in 3% sodium hypochlorite and allowed to dry. Twenty tubers of each cultivar were inoculated approximately halfway between rose and heel ends by injecting 0.2 cm^3 of the spore suspension 8mm into the tissue with a Socorex 2-187 self refilling type syringe. Tubers were incubated in paper bags and kept at 25 ± 2°C and 50-70% RH to promote dry rot development. After a 3 week incubation period, tubers were cut in half at the inoculation site and the extent of dry rot development was determined according to a disease index scale used by Theron & Holz (1989). A randomized block design with three replications was used.

Production of fusaric acid in culture filtrates. Single plugs (1mm^3) from the same mycelium as used in the virulence tests, were used to inoculate Erlenmeyer flasks containing 30 ml culture medium were. The culture medium contained the following: 0·74 g $MgSO_4.7H_2O$; 0.045 g $MnSO_4.4H_2O$; 0.018 g $ZnSO_4.7H_2O$; 5.O µg $CuSO_4.5H_20$; 0.84 g $FeSO_4.7H_20$; 0.11 g Na_2EDTA; 0.10 g myo-inositol; 2.0 mg glycine; 0.5 mg nicotinic acid; 0.5 mg pyridoxine. HC1; 0.10 mg thiamine. HCI; 10.0 g glucose; 2.0 g L-asparagine; 1.0 g KH_2PO_4, 5.0 µg biotin and 1 000 ml distilled water. The flasks were incubated at 22 ± 1°C in the dark on an orbital shaker (50 r.p.m.) for 20 days after which fusaric acid was extracted.

Fusaric acid preparation and extraction. Crude filtrates and a culture medium control were acidified with 1N HC1 and then extracted with an equal volume of ethyl acetate. Extraction efficiency was determined by spiking half of the sample with 0.05 mg ml^{-1} pure fusaric acid (5 - butyl picolinic acid) obtained from Sigma Chemical Company (USA) prior to acidification. The organic phase was removed and dried under vacuum; the residues dissolved in 5ml methanol and stored at - 20°C. Twelve replicates of each isolate were extracted.

Analysis of fusaric acid in the culture filtrates. The culture filtrate was analyzed by HPLC (High Performance Liquid Chromatography). A 50 µl sample of culture filtrate was eluted isocratically from a reversed phase Bondclone 10 C18 column (10 µm, 300 x 3,9 mm) with 40% methanol and 60% of a aqueous solution of 0,62 mM Na_2EDTA and 2% H_3PO_4 (Julien 1988) at a flow rate of 1 ml per minute. The eluate was monitored at 254nm, using a Beckman System Gold Model 166 UV detector. The flow rate was 1 ml min^{-1}. Standard curves were established with pure fusaric acid. The column was stored in 100% methanol and equilibrated with eluting buffer 1 h →1 h before use. Samples were injected as methanol solutions.

RESULTS AND DISCUSSION

Virulence of isolates. The virulence of the groups used in the test (Table 7.1) corresponded with the virulence ranking order that D.J. Theron (personal communication) granted the isolates. From data in Table 7.2 it is shown that the *Fusarium oxysporum* isolates had the largest contribution to the total variance, i.e. 62,7% against the 19,2% of the cultivars used and 11,7 of the cultivar isolate interaction. Both the factors and the interaction of the factors were statistically significant ($P<0,001$).

Results obtained from the virulence tests showed that the cultivars differed in their tolerance against *Fusarium* dry rot. 'Late Harvest' (0.43) was the most tolerant with 'Kimberley Choice' (1.14) mildly susceptible and 'BP1' (1.55) the most susceptible cultivar ($p<0,001$). Values in brackets are the mean dry rot index value each cultivar obtained after inoculation with the *F. oxysporum* isolates. These results correspond with results obtained by Steyn *et al.*, (1991). It is also apparent that the ranking order of the *F. oxysporum* isolates with the different cultivars corresponded to a high degree with the mean value of the ranking order (Table 7.1) and this once again supports the virulence ranking order that D.J. Theron (personal communication) granted the *F. oxysporum* isolates.

Standard curves of fusaric acid. Fusaric acid eluted in a reproducible manner as a sharp peak with an elution time of 5.6 min and a temperature of 20±1 °C.

Efficiency of fusaric acid extraction. Pure fusaric acid co-injected with the sample increased the height of the peak and the product eluted at this peak had the characteristic UV spectrum of fusaric acid. The extraction efficiency (% fusaric acid that was recovered) was determined by spiking half of the sample with 0.05 mg/ml pure fusaric acid. 96.02% fusaric acid was recovered with a coefficient of variance (CV) of 2.0%. All measurements were adjusted accordingly.

Concentration of fusaric acid in culture filtrates of *F. oxysporum* isolates. The concentration of fusaric acid in the culture filtrates of *F. oxysporum* are given in Table 7.1.
Of the variance obtained 99.8% was explained by the *F. oxysporum* isolates ($p<0,001$). The remaining 0.2% was expressed in the residual as error. Differences in fusaric acid production between most isolates was statistically significant (Table 7.1). It is apparent that the five non virulent isolates had the lowest ranking order, which corresponded with the virulence obtained in the pathogenicity test.

To determine whether the fusaric acid concentration obtained in the culture filtrates of the *F. oxysporum* isolates influenced dry rot expression, the correlation was determined between dry rot expression in the three different cultivars respectively and the mean dry rot expression of the three cultivars (Table 7.1). It was found that the linear regression (fusaric acid concentration against dry rot disease index value) of the mean value for all thee cultivars, or for the cultivars 'BP1', and 'Kimberley Choice' fitted significantly ($p = 0.01$) with R^2 60.2%, 69.0% and 66.0% respectively (Figure 7.1). The linear regression of 'Late Harvest' could not be fitted significantly (Figure 7.1) and the correlation was weak ($R^2 = 28.15\%$). This can be explained by the fact that 'Late Harvest' is a tolerant cultivar and that the respective *F. oxysporum* isolates had little effect on this cultivar. Fusaric acid concentration in the potato tubers after inoculation was not determined. It can therefore not be assumed that less dry rot was obtained at the same fusaric acid concentration *in vitro*.

CONCLUSION

The cultivars differed in their tolerance against *Fusarium oxysporum*. The ranking order of cultivars corresponded with ranking orders previously reported. Differences in fusaric acid production by the different isolates was observed. Fusaric acid concentration may contribute to

the virulence of the pathogen. From the results it is evident that fusaric acid production by *F. oxysporum* isolates seems to play a major role in the development of dry rot in potato tubers of susceptible cultivars.

Acknowledgment

We wish to thank Niël Theron for supplying the *F. oxysporum* isolates and Susan Steyn and Annatjie Msiza for their assistance with the extractions.

TABLE 7.1. Average dry rot development in tubers of 'BP1', 'Kimberley Choice' (KC) and 'Late Harvest' (LH), inoculated with *F. oxysporum* isolates as well as concentration of fusaric acid production in culture filtrates of 12 different isolates *in vitro* after twenty days.

	Mean dry rot disease index[1]				Fusaric acid concentrate
Isolates	**BP 1**	**LH**	**KC**	**Average**	**(mg/ml)**
H_2O	0,069 a	0,000 a	0,000 a	0,022 a	0,000 a
N6D10/2(3)	0,067 a	0,000 a	0,069 a	0,044 a	0,019 b
N7P5/2(5)	0,267 ab	0,034 a	0,000 a	0,090 a	0,029 e
WNNR 1174	0,481 ab	0,000 a	0,107 a	0,222 a	0,024 d
N6P7/2(3)	0,680 ab	0,033 a	0,103 a	0,273 a	0,019 b
N6P16/2(5)	0,759 b	0,067 ab	0,233 a	0,356 a	0,022 c
F.o. 7	1,885 c	0,741 cde	1, 423 b	1,352 b	0,033 g
F.o. 1	2,536 d	0,138 abc	1, 846 bc	1,508 bc	0,053 k
F.o. 4	2,609 d	0,423 abcd	2,500 c	1,812 bcd	0,048 I
F.o. 2	2,500 cd	0,828 de	2,115 bc	1,825 bcd	0,032 f
F.o. 5	2,923 d	0,696 bcde	2,286 c	1,848 bcd	0,038 h
F.o. 6	2,385 cd	1,217 e	2, 000 bc	1,889 cd	0,033 g
F.o. 8	2,864 d	1,333 e	2, 577 c	2,261 d	0,051 j

[1] Disease index: 0 = no lesion development; 5 = tuber completely decayed

[2] Fusaric acid concentration (mg) twenty days after inoculation with *F. oxysporum* isolates.

TABLE 7.2 Analysis of variance of the results obtained from the pathogenetic tests for the three potato cultivars BP1, Kimberley Choice and Late Harvest, inoculated with different *Fusarium oxysporum* isolates.

Source of variation	Degrees of Freedom	Sum of squares	Mean square	F-ratio	p-value
- Main effects					
cultivars	14	106,31	7,59	71,14	<0,00
F. oxysporum. isolates	2	24,88	12,44	116,53	1
- Interaction	12	81,44	6,77	63,57	<0,001
F. oxysporum. cultivar	24	15,23	0,64	5,95	<0,00
- Residual	78	8,33	0,11		1
- Total	116	129,87			

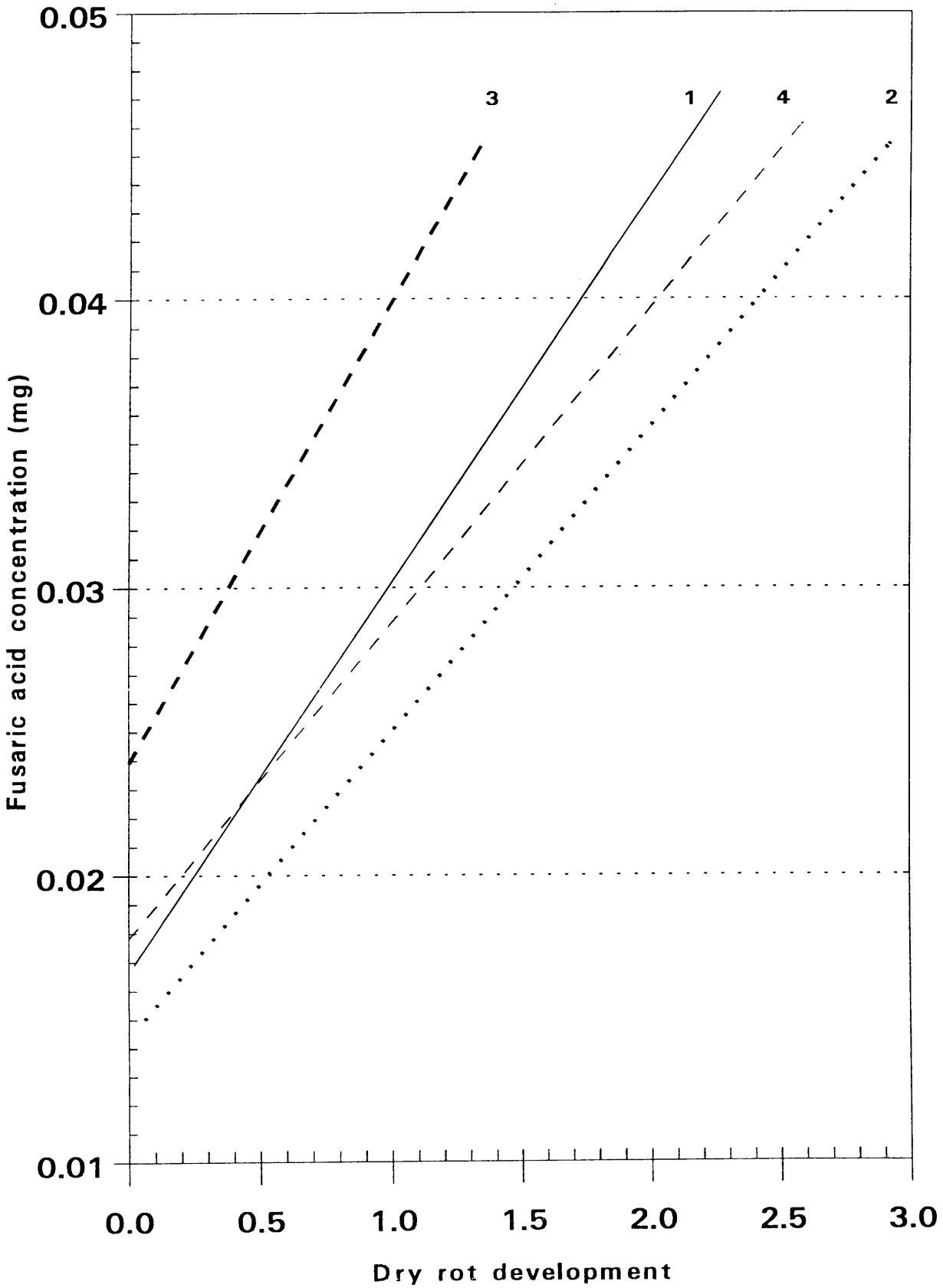

Figure 7.1. The correlation between fusaric acid production (20 days incubation, *in vitro*) of different *Fusarium oxysporum* isolates and dry rot development in potato tubers of three cultivars. (1= combined; 2 = 'BP1'; 3 = 'Late Harvest', 4 = 'Kimberley Choice'

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8. DIFFERENTIATION OF *FUSARIUM OXYSPORUM* ISOLATES ON THE BASIS OF VEGETATIVE COMPATIBILITY, VIRULENCE AND RAPD's*

INTRODUCTION

Fusarium oxysporum W.C. Snyder & H.N. Hans, causes dry rot, stem-end rot and wilt of potatoes (*Solanum tuberosum* L.) (Nelson *et al.*, 1981). For the breeding of Fusarium-resistant potato cultivars, a reliable identification method that distinguishes between the different *F. oxysporum* isolates that cause the disease is needed.

Snyder & Hansen (1940) grouped strains of *F. oxysporum* into formae speciales on the basis of their host specificity. With few exceptions, strains of the fungus, whether from the same or different formae speciales, are morphologically indistinguisible. Formae speciales have been subdivided into physiological races that attack only certain cultivars of a host species. Puhalla (1985) showed that the vegetative compatibility group (VCG) may be a handy tool for differentiating forma speciales of *F. oxysporum.* Fungal strains that are able to anastomose (form heterokaryons) with each other are considered vegetatively compatible and are assigned to a single VCG. Puhalla (1985) first used auxotrophic, nitrate-non utilizing (*nit*) mutants to establish a vegetative compatibility system in *F. oxysporum*. *Nit* mutants can be readily recovered in high frequencies by selecting for chlorate resistance (Correll *et al.*, 1985; Correll *et al.*, 1986b; Elias & Schneider, 1986; Elmer & Stephens, 1986; Bosland & Williams, 1987; Jacobson & Gordon, 1988; Harveson & Rush, 1997). Puhalla (1985) placed 21 strains of *F. oxysporum* into 16 VCG's. A correlation between VCG and formae speciales was observed.

Correll *et al.*, (1987) refined the heterokaryon technique by demonstrating that *nit* mutants of *F. oxysporum* could be subdivided into at least three phenotypic classes. These classes could be differentiated by the ability of *nit* mutants to use various nitrogen sources. *F.*

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oxysporum has no known teleomorph, and genetic analysis is therefore not possible. Puhalla & Spieth (1985) and Klittich & Leslie (1988) discovered similar phenotypes among *nit* mutants of *F. moniliforme* J. Sheld., a closely related species with a teleomorph. Two of the phenotypic classes of *F. moniliforme* were single locus mutations of the gene for nitrate reductase and for the pathway-specific regulatory gene for nitrite reductase. These phenotypes were labelled with the genotype designation *nit* 1 and *nit* 3, respectively (Klittich *et al.*, 1986; Yoder et al., 1986; Klittich & Leslie, 1988). The third phenotype in *F. moniliforme* mapped to one of five loci required for the synthesis of the molybdenum cofactor, which is necessary for nitrate reduction and purine dehydrogenase (Marzlut, 1981; Klittich & Leslie, 1988). This phenotype was described in *Aspergillus nidulans* G. Wint (Cove, 1976) and was called *Nit* mutants. Correll *et al.*, (1987) presumed similar mutations were likely to exist among the classes of *nit* mutants in *F. oxysporum*. Although clear relationships between pathogenicity and VCG's have been found in several instances (Correl *et al.*, 1986a & b; Bosland & Williams, 1987; Kistler, *et al.*, 1987; Katan & Katan, 1988) more complex associations were found in other instances (Puhalla & Hummel, 1983, Jacobson & Gordon, 1988; Correl, 1991; Elias & Schneider, 1991). No relationship was found between VCG's and pathogenicity in other instances (Elmer & Stephens, 1989). Random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) offers several advantages that may be useful in studying formae speciales and races of *F. oxysporum*. RAPD reduces the time needed for race identification in diseased plants and provides genetic information on isolates studied (Grajal-Martin *et al.*, 1993; Assigbetse *et al.*, 1994 Amoah *et al.*, 1996). RAPD has several advantages over other polymorphic DNA-detecting techniques, including RFLP (Restriction fragment length polymorphism). These advantages include quickness, small amounts of template DNA needed, no requirement of DNA sequence information and no need for radio-active materials (Grajal-Martin *et al.*, 1993; Amoah *et al.*, 1996).

Manulis *et al.*, (1994) used the RAPD technique for identification of *Fusarium oxysporum* f. sp. *dianthi* from carnation and found that compared with other methods of identifying *F. oxysporum* f. sp. *dianthi*, the RAPD procedure was simple, rapid and reproducible. Various other authors used the RAPD technique for studying different formae speciales of *F. oxysporum* (Whitehead *et al.*, 1992, Grajal-Martin *et al.*, 1993; Woudt *et al.*, 1995

Achenbach & Patrick, 1996).

The aim of this study was to evaluate virulence, vegetative compatibility tests and the RAPD technique for their usefulness in differentiating among isolates of *Fusarium oxysporum* that cause dry rot of potatoes.

MATERIALS AND METHODS

Isolates : Twelve isolates of *Fusarium oxysporum* which had been isolated from potato tubers with dry rot symptoms were used. Their virulence had previously been verified by Theron & Holz (1989). The isolates differed in their ability to cause disease symptoms.

Virulence tests : Conidial suspensions of the twelve isolates were prepared as described by Venter et al., (1992). Unblemished potato tubers of cultivars 'BP1', 'Kimberley Choice' and 'Late Harvest' were disinfected for 15 min in 3 % sodium hypochlorite and allowed to dry. Twenty tubers of each cultivar were inoculated as described by Venter *et al.*, (1992; 1996).

Vegetative compatibility of generation of nit mutants. Chlorate-resistant mutants were generated from the 12 *F. oxysporum* isolates on potato-sucrose chlorate medium (KPS) and minimal chlorate medium (KMM) as well as on four alternative media: potato-dextrose chlorate medium (PDC) (Correl *et al.*, 1987) malt chlorate agar (MA), cornmeal chlorate agar (CMA), and Czapek-Dox chlorate agar (CDA) all amended with potassium chlorate (15 g/L). The chlorate-resistant mutants obtained were grown on minimal medium (MM) (Puhalla, 1985) containing only nitrate as the nitrogen source, which tested for a deficiency in the nitrate reduction pathway. Mutants that grew on MM as thin expansive colonies with no aerial mycelium were considered *nit* mutants. These mutants showed wild-type growth on complete medium (CM) (Correl *et al.*, 1987).

Vegetative compatibility of nit mutant phenotypes. The physiological phenotypic classes of all nit mutants were determined by colony morphology on media containing one of five different nitrogen sources (nitrate, nitrite, hypoxanthine, ammonium, and uric acid)

(Correl *et al.*, 1987*)*. The *nit* mutants were assigned to three phenotypic classes. These classes presumably represent a mutation at either a nitrate reductase structural locus (*nitl*), a nitrate assimilation pathway-specific regulatory locus (*nit3)*, or at least five loci that affect the assembly of a molybdenum-containing cofactor that is necessary for nitrate reductase (EC 1.6.6.6.) activity (NitM). To distinguish nitrite reductase mutants from pathway-specific regulatory mutants, we evaluated *nit mutants* that grew as thin colonies on nitrite medium for nitrate reductase activity by testing for nitrite excretion as described by Cove (1976).

Vegetative compatibility groups. All *nit* mutants generated from a single isolate were paired in genetic complementation tests. All the *nit* mutants recovered from the same parent were paired with at least one nit 1, one *nit3*, and one *NitM* mutant from that parent (correl *et al,* 1987). Genetic complementation was indicated by the presence of dense aerial growth where the mycelia of two *nit* mutant colonies came in contact and formed a hetrokaryon. A *nitl, nit3,* and Nit M mutant from each isolate was paired in all possible combinations for the determination of vegetative compatibility groups.

RAPD technique

DNA extraction

Fungal cultures were grown in potato-dextrose broth for 7 days at 24±1°C on an orbital shaker at 250 rpm. The mycelium was filtered through cheesecloth, washed with sterile water and transferred to filter paper for removal of excess water; 0.5 to 1.0 g of wet mycelium was ground into a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted according to a modified version of the method described by Edwards *et al.*, (1991). Carborandum (400 Grit) was added to the powder and it was further grounded with a glass grinder and then incubated at 60°C for 30 min in 400 µℓ prewarmed supaquick buffer (200 mM Tris-HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5 % SDS). An equal volume of chloroform: isoamylalcohol was added and mixed well for 5 min by inverting the tube several times. After mixing it was centrifued at 10 000 x g for 10 min at 22 ± 2°C. Supernatant (350 µℓ) was transferred into a clean microfuge tube, 0.6

volumes of ice cold isopropanol was added, mixed by gentle invertion and left at -20°C for 30 min to precipitate. This aqueous mixture was centrifuged at 10 000 x g for 10 min at 4°C and then washed with 70 % ethanol. A digital fluorometer (Sequoia-Turner, model 450) was used to determine the DNA concentrations.

PCR conditions

PCR was carried out in 10 µℓ of a solution containing 10 ng *F. oxysporum* genomic DNA, 100 mM Tris-HCl, pH 8.8, 500 mM KCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 0.1 mM each of dNTP (Boehringer Mannheim Germany), 1 µl of primer and 0.5 units/µℓ DyNA Zyme™ II under 20 µℓ of mineral oil. Amplification was performed in a thermal cycler (Hybaid, Omni Gene) programmed for one cycle of 90 s at 94,5 °C, followed by 35 cycles of 20 s at 94.5 °C, 30 s at 37 °C and 45 s at 72 °C as well as a final elongation cycle of 3 min at 72°C. After PCR, 6 µℓ of the products were separated according to size in a 1 % agarose gel (Boehringer Mannheim, Germany) run in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) and visualised by UV light after ethidium bromide staining.

The four primers (Table 1) were described by Demeke et al., (1993) and synthesized by the University of Natal (Durban, South Africa). All reactions were repeated at least 3 times and only repeatable results were taken into consideration.

Data analysis

RAPD profiles were scored visually on the basis of the presence (1) or absence (0) of each band for all genotypes. Amplifications were performed three times, and only reproducible amplification products were included in the data analysis. Pairwise comparisons were made between genotypes, on the basis of bands that showed polymorphisms among all genotypes, and the values used to generate Jaccard's similarity coefficiets (Jaccard, 1908). Cluster analysis, using the unweighted pairgroup method with arithmetical average (UPGMA) (Sneath & Sokal, 1973) was performed with the NTSYS-pc program (Numerical Taxonomy and Multivariate Analysis System; version 1.8, Exeter Software,

Setauket, N.Y.). These results were used to generate a dendogram displaying the hierarchical associations among all genotypes (Figure 8.2). The cophenetic value matrix was computed and compared with the similarity matrix in order to calculate the cophenetic correlation.

TABLE 8.1: Sequence of primers used in the study

Code				
116	TAC	GAT	GAC	G
131	GAA	ACA	GCG	T
153	GAG	TCA	CGA	G
184	CAA	ACG	GCA	G

RESULTS AND DISCUSSION

Virulence of isolates. The virulence of the isolates (Table 8.2) were discussed by Venter & Steyn (1997).

Results obtained from the virulence tests showed that the cultivars differed in their tolerance against *Fusarium* dry rot. 'Late Harvest' was the most tolerant with 'Kimberley Choice' mildly susceptible and 'BP1' the most susceptible cultivar ($P<0,001$) (Venter & Steyn, 1997). These results correspond with results obtained by Steyn et al., (1991). It is apparent that the ranking order of the *F. oxysporum* isolates with the different cultivars corresponded to a high degree with the mean value of the ranking order (Table 8.2).

Vegetative compatibility tests

Recovery of *nit* mutants. Isolates of *F. oxysporum* f.sp. *tuberosi* readily formed chlorate-resistant sectors on the six chlorate-containing media. All the isolates were more inhibited on minimal chlorate medium(KMM) than on Potato sucrose chlorate medium (KPS). The average number of sectors per petri dish per isolate on KMM, malt agar

(MA) and maize meal agar (CMA) ranged from 1.1 to 4.2. Less than 25% of the isolates developed more than two sectors, and only 20% developed two or more phenotypically different *nit* mutants. On KPS and Potato dextrose chlorate agar (PDC) there were between 1.7 and 5.5 sectors per isolate (Figure 8.3). Of all the isolates, 83% developed at least two phenotypically different *nit* mutants on KPS and PDC. Of these isolates, 65% developed two or more phenotypically different *nit* mutants. Not all the isolates formed *nit* mutants on KPS, MA, CMA and Czapet box chlorate agar (CDA). On KMM and PDC, all the isolates generated at least one *nit* mutant. Thus, KMM and PDC were used in all the other tests. These results correlated with results found by Venter *et al.*, (1992). All *nit* mutants were evaluated to determine the physiological phenotypic classes.

***Nit* mutant phenotypes.** All the *nit* mutants obtained from the *F. oxysporum* isolates could be divided into three phenotypic classes. The majority of *nit* mutants were *nit* 1 mutants. The frequency of *nit* 1 mutants was higher on PDC than on KMM, whereas the frequencies of *nit 3* and *Nit*M mutants were higher on KMM than on PDC. A few sectors that were resistant to chlorate were recovered, but they had a wild-type colony morphology om MM. The frequency of these wild-type mutants was higher on PDC than KMM. At least one *Nit*M mutant was found for every isolate.

***Nit* mutant complementation tests.** Complementation between different *nit* mutants was indicated by the development of dense aerial growth where the mycelia of the colonies grew together and anastomosed. When *Nit*M mutants were paired (or were involved in the pairing), complementation occurred more rapidly and resulted in heterokaryons that were more robust than those of other *nit* mutant pairs (Figure 8.4). When *nit* 1 and *nit* 3 mutants were paired, weak vegetative compatibility reactions were obtained. When *nit* and *nit* 3 mutants were paired, heterokaryons were not obtained within the first 3 weeks, and after 3 weeks the compatibility reactions were weak. In some isolates, no compatibility reactions were observed even after 4 weeks. These results correlated with results of a previous study (Venter *et al.*, 1992).

Vegetative compatibility groups. The 12 isolates were assigned to 3 vegetative compatibility groups (VCG's). Isolates F.o.2, F.o.4, F.o.5, F.o.6 and F.o.7 were in VCG

F which was previously described by Venter et al., (1992) and Isolates N6 D10/2(3), N7 P5/2(5) and N6 P7/2 (3) were in VCG E (Venter *et al.*, 1992). Isolate WNNR 1174 was incompatible with the other isolates and could not be assigned to a specific VCG. Isolates F.o.1, F.o.8 and N6 P16/2(5) were assigned to VCG I, a new VCG for isolates in South Africa.

The most virulent isolates were in VCG F except for isolates F.o.1 and F.o.8 in VCG I. No single isolate may, however, safely be regarded as representative of the pathogenic (virulence) potential of all isolates. This consideration bears directly on the selection of isolates in screening potato cultivars for resistance to Fusarium dry rot. It seems as though an isolate(s) belonging to VCG F and/or VCG I may be used for screening of cultivars. Pathogenicity tests (and/or virulence tests), although time-consuming and influenced by temperature, humidity and pH have been, and undoubtedly will continue to be, useful for the characerization of isolates. Finding other techniques that are less time-consuming and viariable than pathogenicity tests would also be useful in differentiating *F. oxysporum* isolates, which apparently are a genetically diverse group unified by the common trait of pathogenicity to potatoes. In other studies VCG's have been useful in identifying sub-specific groups within a morphological species (Croft & Jinks, 1977; Anderson, 1982; Puhalla & Hummel, 1983; Sidhu, 1986; Venter *et al.*, 1992).

RAPD technique

To select primers that generate informative arrays of PCR products four primers were tested with the DNA of 12 isolates of *F. oxysporum*. The RAPD patterns differed with each of the primers. Some of the primers generated more DNA fragments than did others. The size of the products was within the range of 2 000 to 150 bp. All four primers gave reproducible PCR patterns. Only major amplification products were considered. PCR's were carried out with each of the four primers and DNA from the 12 isolates.

To examine the reproducibility of the RAPD patterns DNA preparations of 5 different cultures of every isolate were used. Identical patterns were obtained with all the primers tested.

Since it's introduction (Welsh & McClelland, 1990 and Williams *et al.*, 1990) the RAPD technique has been widely used to detect genetic polymorphisms in various organisms including fungi (Guthrie et al., 1992; Schafer & Worstemeyer, 1992). The four primers used in this study clearly distinguished the twelve *F. oxysporum* isolates (Figure 8.1). A dendrogram with branch lengths proportional to the differences among isolates was constructed (r = 0,84) (Figure 8.2). The relationships depicted in the dendrogram indicate that the *F. oxysporum* isolates were differentiated into two major groups which on their turn could be differentiated into two smaller groups. Isolates N6 D10/2(3), N7 P5/2(5) and N6 P7/2(3) which are all assigned to VCG E are in the first RAPD Group (Table 8.1) although Isolate N6 D10/2(3) is different from the other two.

Isolate WNNR 1174 was discrete from the other isolates, forming a separate group. This also correlated with results obtained by VCG grouping. Isolate N6 P16/2(5) in VCG I was closely related to the isolates in VCG E and Isolate F.0.8 in VCG I was closely related to isolates in VCG F. These two isolates could not be assigned to a single RAPD grouping. Isolates from VCG F were assigned to the second major RAPD Group, although two subgroups are apparent. Isolates F.0.1, F.0.2, F.0.4 and F.0.5 are more closely related to each other than the isolates in the other subgroup (F.0.6 and F.0.7). What is important however is that the pathogenicity, VCG grouping and RAPD results have a similar pattern.

Results in this study show that vegetative compatibility and RAPD could be useful for the plant pathologist and plant breeder who wants to distinguish between isolates that cause dry rot of potato in order to be able to determine which isolates to use during screening of potatoes for improved resistance.

Non-pathogenic strains of *F. oxysporum* are morphologically indistinguishable from the pathogenic strains. Pathogenicity assays are expensive, time-consuming and not suitable for screening large numbers of isolates as part of resistance - breeding programme. In this study three methods were used to characterize isolates recovered from potato i.e. pathogenicity, VCG and RAPD analysis. Variation was found within all of these methods. It was however possible to group the isolates. The VCG and RAPD data provided a

meaningful grouping of the isolates in terms of the pathogenicity of the isolates. These results were in correspondence with results obtained by Woudt *et al.*, (1995) with *F. oxysporum* from *Cyclamen*; Achenbach & Patrick (1996) with *Fusarium solani* causing soybean Sudden Death Syndrome and Whitehead *et al.*, (1992) with *F. oxysporum* f.sp. *pisi*. Although there was a correlation between VCG and RAPD grouping, the correlation was not precise and this is not unusual for *F. oxysporum.*

In *F. oxysporum* f.sp. *melonis*, Jacobson & Gordon (1988; 1990) found that a single VCG may contain more than one race and that a race may occur in more than one VCG. This is the case with VCG I in this study. This was also found in *F. oxysporum* f.sp. *cubense* (Ploetz, 1990) and *F. oxysporum* f.sp. *lycopersici* (Elias & Schneider, 1991). An exception is *F. oxysporum* f.sp. *pisi* (Whitehead *et al.*, 1992) where the correlation between VCG and RFLP was precise and *F. oxysporum* f.sp. *dianthi* (Baayen & Klein, 1989) where each race corresponded exactly with a VCG. In conclusion, the VCG and RAPD methodologies used in this study have classified *F. oxysporum* isolates that cause dry rot on potatoes into groupings that coincide with races and due to cost-effectivity RAPD analysis may be the more widely applicable technique, especially for screening large samples of isolates.

Table 8.2. Vegetative compatibility groups of 12 isolates of *Fusarium oxysporum* and average dry rot development in tubers of potato cultivars BP1, Kimberley Choice (KC) and Late Harvest (LH), inoculated with these isolates. Values with the same symbol are not statistically different.

Isolates	Mean dry rot disease index[1a]				VCG GROUP[b]	RAPD GROUP
	BP 1	LH	KC	Average		
H_2O	0,069 a	0,000 a	0,000 a	0,022 a	-	
N6D10/2(3)	0,067 a	0,000 a	0,069 a	0,044 a	E	I A
N7P5/2(5)	0,267 ab	0,034 a	0,000 a	0,090 a	E	I B
WNNR 1174	0,481 ab	0,000 a	0,107 a	0,222 a	-	II
N6P7/2(3)	0,680 ab	0,033 a	0,103 a	0,273 a	E	I B
N6P16/2(5)	0,759 b	0,067 ab	0,233 a	0,356 a	I	I B
F.o. 7	1,885 c	0,741 cde	1, 423 b	1,352 b	F	III B
F.o. 1	2,536 d	0,138 abc	1, 846 bc	1,508 bc	I	III A
F.o. 4	2,609 d	0,423 abcd	2,500 c	1,812 bcd	F	III A
F.o. 2	2,500 cd	0,828 de	2,115 bc	1,825 bcd	F	III A
F.o. 5	2,923 d	0,696 bcde	2,286 c	1,848 bcd	F	III A
F.o. 6	2,385 cd	1,217 e	2, 000 bc	1,889 cd	F	III B
F.o. 8	2,864 d	1,333 e	2, 577 c	2,261 d	I	III A

[1] Disease index: 0 = no lesion development; 5 = tuber completely decayed

[a] Results obtained by Venter & Steyn (1997)

[b] VCG groups E and F previously described by Venter *et al.*, 1992)

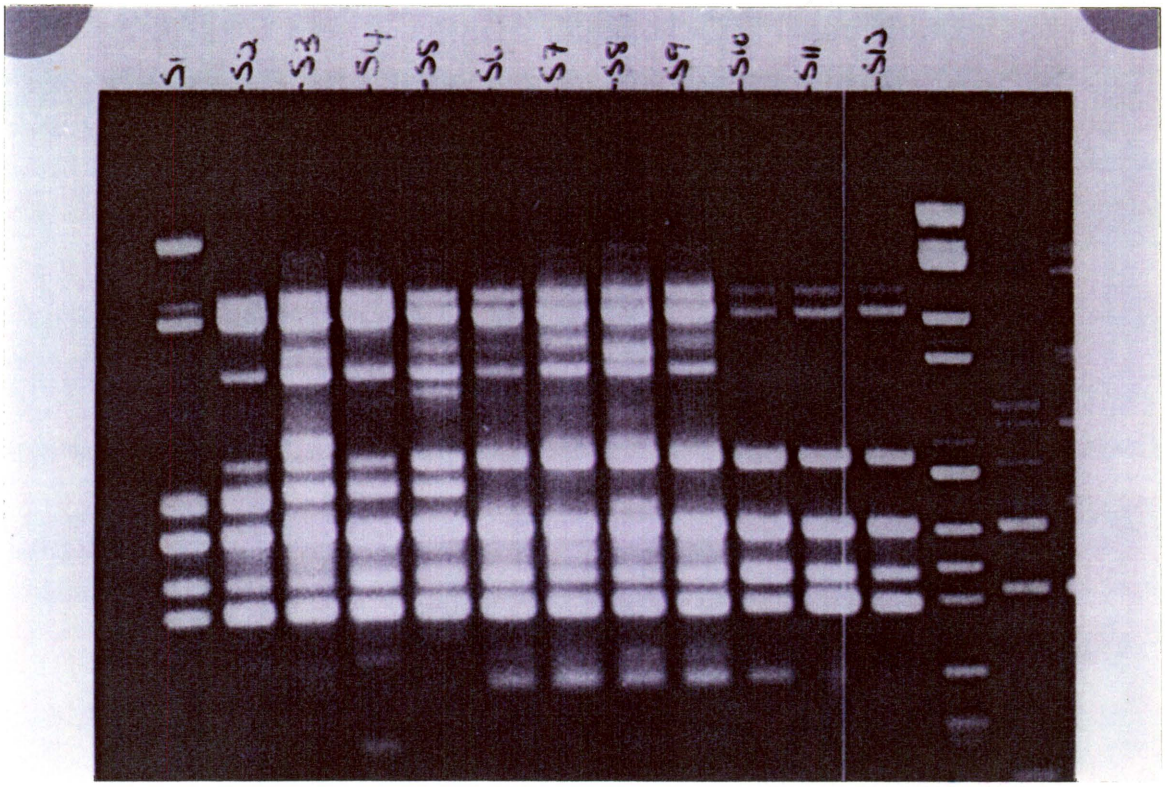

Figure 8.1: Random amplified polymorphic DNA pattern obtained by amplification of DNA from twelve *Fusarium oxysporum* isolates amplification with primer, Dem 116 (Table 8.1)
lane 1: isolate N6 D10/2(3), lane 2: N7 P5/2(5), lane 3: WNNR 1174, lane 4: N6 P7/2/3, lane 5: N6P16/2/5, lane 6: F.O.1., lane 7:F.O.2., lane 8: F.O.4., lane 9: F.O.5., lane 10: F.0.6, lane 11: F.0.7., lane 12: F.0.8., Lane 13: 1-kb marker

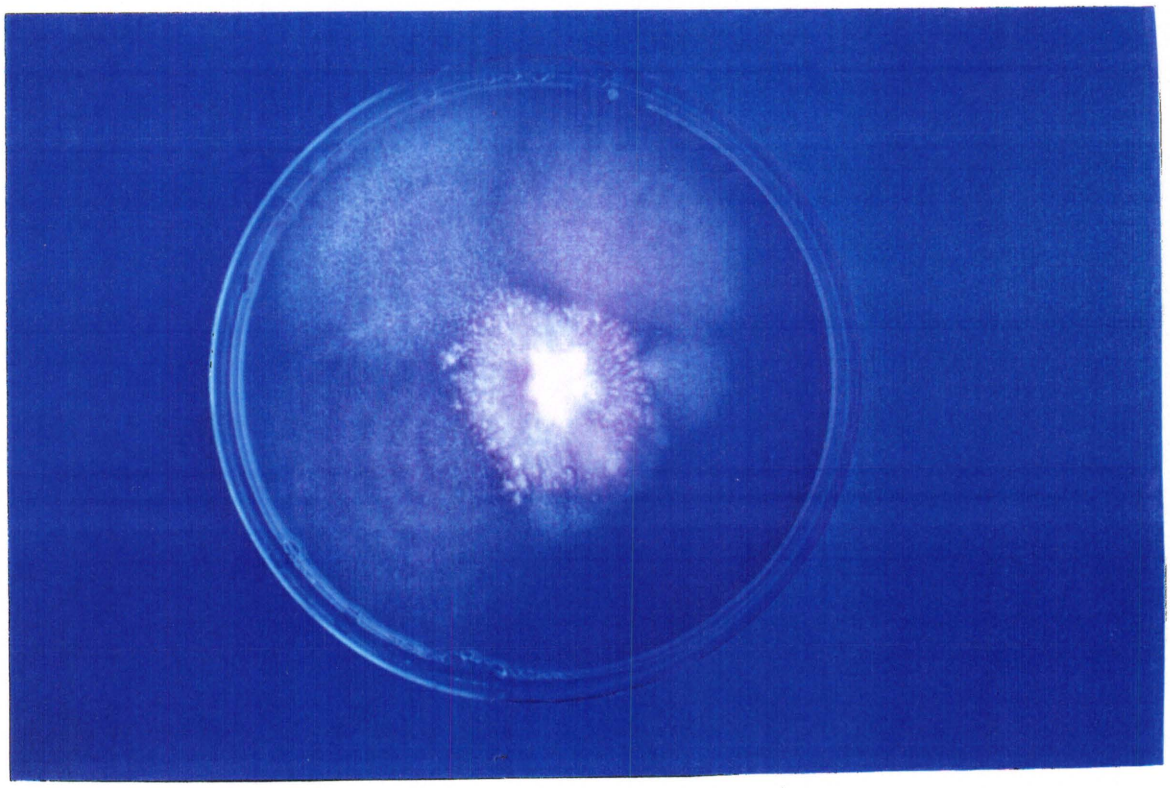

Figure 8.3: Chlorate resistant sectors of *Fusarium oxysporum* (F.0.1) on the chlorate containing mediums KPS (Potato Sucrose chlorate medium)

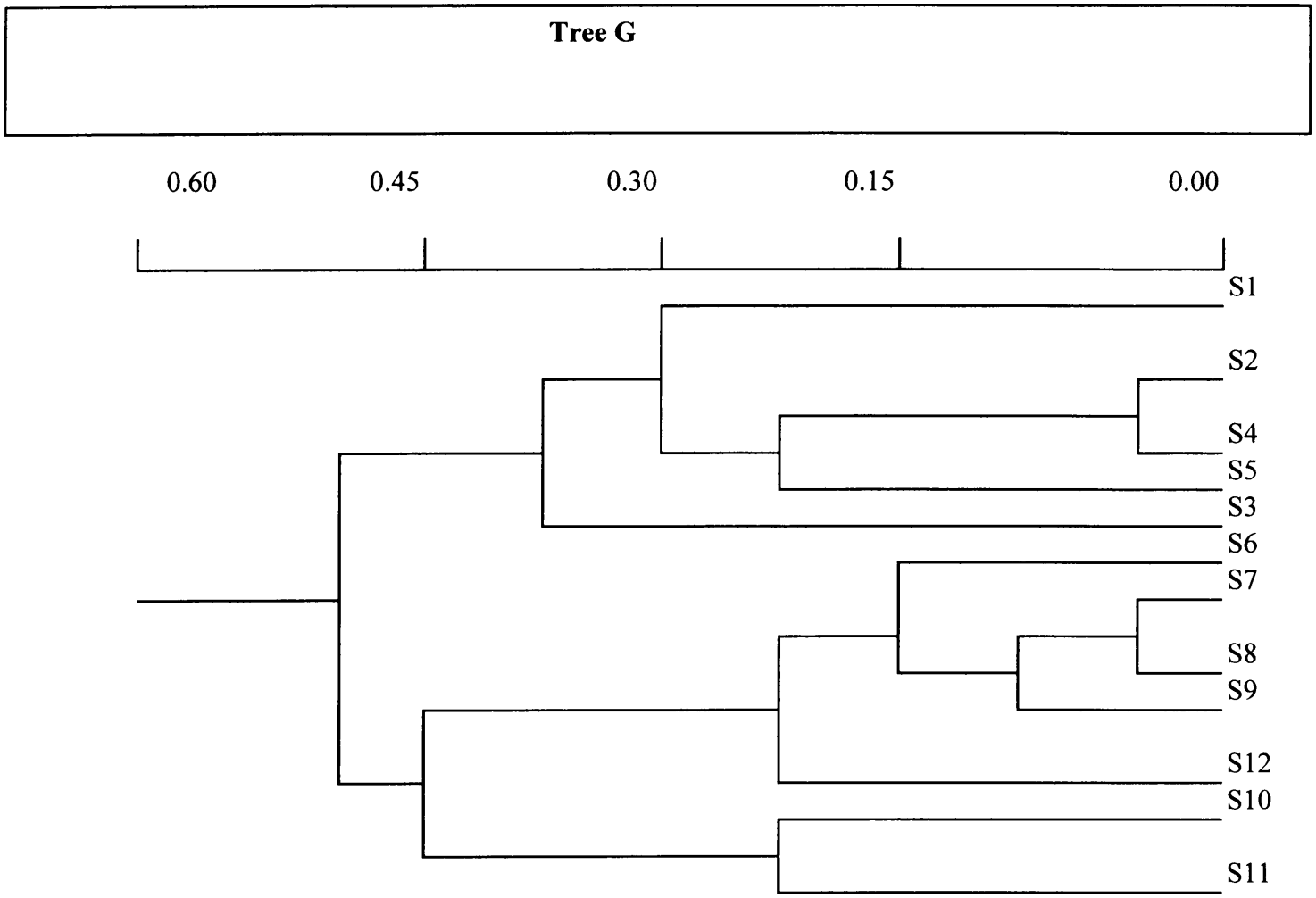

Figure 8.2 Dendogram showing the relationship of the *Fusarium oxysporum* isolates that cause potato dry rot. Genetic distances were computed from data generated by four random amplified polymorphic DNA primers. S1 = N6 D10\2 (3); S2 = N7 P5\2 (5); S3 = WNNR 1174; S4 = N6 P7\2 (3); S5 = N6 P16\2(5); S6 = F.0.1; S7 = F.0.2; S8 = F.0.4; S9 = F.0.5; S10 = F.0.6; S11 = F.0.7; S12 = F.0.8.

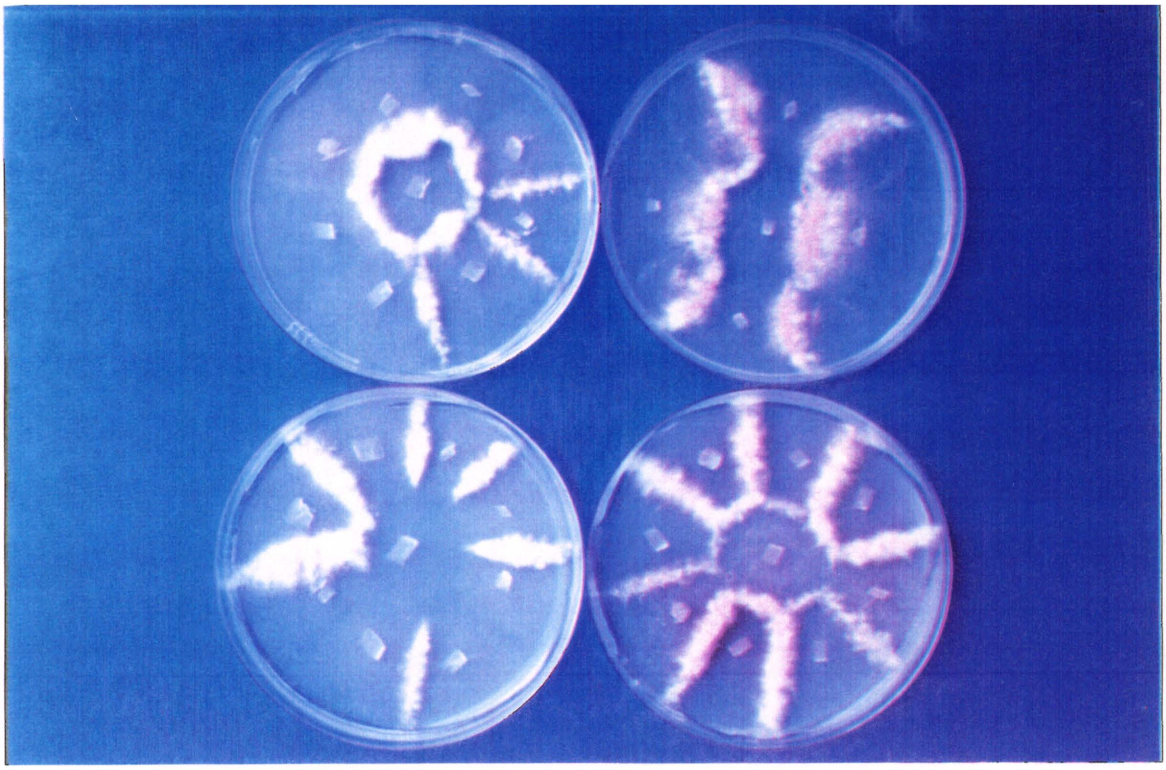

Figure 8.4: Vegetative compatibility reaction between *nit* mutants of *Fusarium oxysporum*

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9. *IN VITRO* SELECTION FOR FUSARIC ACID RESISTANT POTATO PLANTS

INTRODUCTION

Plant tissue culture has in recent years attracted growing interest from plant pathologists and breeders aiming for disease resistance, since it could meet the requirements for screening a large number of individuals in a small space. Large-scale screening of cell populations using *in vitro* procedures successfully produced resistant plants against pathotoxins (Wenzel, 1985).

In vitro selection has been successfully applied by Gengenbach & Green (1975) for the selection of *Helminthosporium* resistant corn plants. Since then the technique was used frequently, also aimed at the selection of *Fusarium* resistance in various crops (Behnke, 1980; Hartman *et al.*, 1984; Arcioni *et al.*, 1987; Chawla & Wenzel, 1987a,b). In spite of many efforts, incidental successes have been achieved (Löffler & Mouris, 1992). Wenzel & Fouroughi-Wehr (1990) tested progenies of barley plants which were selected *in vitro* for resistance against fusaric acid. They did not find any segregation for resistance against fusaric acid. Moreover, the level of resistance of the progeny equalled that of the non-selected control. They concluded that too little genetic change due to somaclonal variation occurred. In contrast, Heath-Pagliuso & Rappaport (1990) confirmed with genetic studies of regenerated plants that resistance against *Fusarium* was induced in the callus tissue of celery by somaclonal variation. Resistant plants have been obtained in two ways : by selecting cell lines resistant to purified or crude toxin(s) produced by the pathogen followed by regeneration of plants (Hartman *et al.*, 1984; Rines & Luke, 1985; Shahin & Spivey, 1986) or by screening regenerated plants obtained from unselected cell cultures with toxin(s) to identify resistant somaclonal variants (Shepard *et al.*, 1980)

One of the prerequisites for *in vitro* selection is a suitable selective agent, preferably a toxin produced by the pathogen (Löffler & Mouris, 1992).

Phytotoxins have been recognised as useful tools for the induction and selection of disease-resistant plants by using tissue culture (Branchard, 1984; Daub, 1984; Wenzel, 1985). Some phytotoxins are well characterized as single molecules that are easier to use in a selection programme as opposed to the fungal or bacterial pathogens (Mégnégneau & Branchard, 1988). Phytotoxins are generally classified into two major groups : non-specific and host-specific. The genus *Fusarium* is recognised as producing at least two main non-specific wilt toxins, namely fusaric acid and lycomarasmin (Gäuman, 1957, Pouteau-Thouvenot & Barbier, 1966). Lycomarasmin has never been extracted from diseased hosts whereas the former has (Pegg, 1981). Gäuman (1957) showed that important phytopathogenic species of *Fusarium* belonging to the Elegans-group (like *Fusarium oxysporum*) synthesize this toxin. He also explained the pathogenic action of fusaric acid. This toxin was studied extensively in the past (Kern, 1972) and recently because of its potential as selective agent for *in vitro* selection (Julien, 1988; Matsui & Watanabe, 1988; Mégnégneau & Branchard, 1988). However, the role of fusaric acid during pathogenesis, is still not clear (Löffler & Mouris, 1992). Ouchi *et al.*, (1989) demonstrated that tomato plants, infected with fusaric acid degrading *Pseudomonas* bacteria, were protected partially against *Fusarium* wilt caused by *Fusarium oxysporum*. This indicates that fusaric acid might play a role in the pathogenesis and that insensitivity of the plant for this toxin may enhance the resistance against the pathogen. Venter *et al.*, (1996) and Venter & Steyn (1997) found a correlation between fusaric acid production and virulence of isolates of *Fusarium oxysporum* that causes potato dry rot in South Africa.

This paper describes the toxicity of fusaric acid on callus cultures and leaf discs of five potato cultivars with different resistance levels to *Fusarium oxysporum* in order to determine the relationship between susceptibility to *F. oxysporum* and to *fusaric acid.*

MATERIALS AND METHODS

Callus cultures of potato cultivars 'Late Harvest', 'Vanderplank', 'BP1', 'Up-to-Date' and 'Kimberley Choice' were established as described in Chapter 3. For testing lethality of toxin on callus, the callus were divided into pieces 0,5 cm in diameter. Callus were put

into petri dishes (9 cm diameter) containing 0, 0.05, 0.1, 0.15, 0.25, 0.3, 0.35, 0.4 and 0.5 mM fusaric acid in callus-initiating media. Fusaric acid (Sigma Chemical Co, St. Louis) solution was passed through a 0,45 µm Millipore filter and added to luque warm autoclaved callus-initiating medium to obtain the desired concentrations. For each fusaric acid concentration the pH was adjusted before autoclaving (20 min at 121 °C) so as to obtain a pH of 5,7 after fusaric acid addition.

The fresh weight of the callus on each treatment was determined after 4 weeks and the relative fresh mass increase was determined according to the formula :

$$\frac{Xn - Xo}{Xc - Xo} \times 100$$

where Xn = fresh mass for concentration

Xc = fresh mass of the control

Xo = fresh mass of initial inoculum

A dose reaction curve was obtained to determine the lethal concentration of fusaric acid on callus growth and where 90% of the callus died.

Callus which survived the concentration where 90% calli died were selected and to test the resistance of the selected callus lines, callus were maintained on non-toxic media for 3 months (Chawla & Wenzel, 1987a) and were then placed back on the same concentration of toxic medium and non-toxic medium, respectively. Increase in fresh weight was measured after 4 weeks as described before. After this step, callus was transferred to shoot induction medium to regenerate plants (Chapter 4).

A callus bioassay was used to determine the effect of fusaric acid on callus colour. Discolouration was scored using an index described by Löffler & Mouris (1992) which was modified ranging from 0 (healthy, light yellow callus) to 3 (strong discolouration) (Figure 9.2) . Calli were incubated on callus-initiating medium containing 0, 0.05, 0.1, 0.15, 0.25, 0.3, 0.35, 0.4 and 0.5 mM fusaric acid in the dark at 24±1°C. After 4 weeks (30 days) calli were evaluated for growth and colour.

Leaves of selected plants from each progeny were cut and placed on regeneration medium (Chapter 5) containing 0.2 mM fusaric acid for testing the resistance of regenerated plants.

The leaves with the apical and basal 1 - 3mm removed were placed with the adaxial side facing upwards on plates containing the toxic regeneration medium. Leaf discs were subcultured onto fresh toxic medium once a week and when callus production could be visualised on the cut surfaces of the leaves (5 - 8 weeks, depending on the cultivar), the leaves were transferred to regeneration medium without auxin (but with toxin) to stimulate shoot production. Emerged shoots (1 cm in length) were rooted on modified MS-medium (Chapter 4) without toxin.

Another method for testing the resistance of regenerated plants was used. After the shoots had reached a certain size, leaflets were removed and tested for resistance to fusaric acid by immersing leaflets from sterile cultures into a liquid medium containing MS medium with 0,2 mM fusaric acid.

RESULTS AND DISCUSSION

It is well known that fusaric acid is toxic to many prokaryotes and eukaryotes (Marasas *et al.*, 1984), among which many plants. Recently growth of soybean plants was found to be affected by circa 6 µM fusaric acid (Matsui & Watanabe,, 1988) and lily bulbs were found to be affected by 10 µM or higher fusaric acid concentrations (Löffler & Mouris, 1992). Non differentiated callus tissue of lily was also sensitive to fusaric acid. In this paper we confirm the toxicity of fusaric acid to potatoes. Regeneration of plantlets was decreased in the presence of fusaric acid at concentrations of 0.01 mM and higher. The non-differentiated callus tissue of potatoes was sensitive to fusaric acid. A concentration of 0.3 to 0.5 mM fusaric acid in the callus-inducing medium killed callus pieces within 30 days (Figure 9.1). These findings correspond with those of Mégnégneau & Branchard (1988), who demonstrated the inhibition of callus growth of muskmelon at 0.1 mM fusaric acid and those of Jullien (1988) who found a LD_{50} for callus of asparagus at 0.35 mM fusaric acid as well as those of Löffler & Mouris (1992) who found that a concentration of 0.5 mM fusaric acid killed callus of lily within 18 days. Both the callus and leaflet assays of potato can be used to demonstrate the toxic activity of fusaric acid to potatoes. From these assays it was evident that sensitivity is expressed in non-differentiated tissue. This is important for *in vitro* selection with fusaric acid (or any other agent) as a selective

agent. A typical effect of the fusaric acid on callus is the greyish discolouration rather than browning of the callus. This lack of browning may be related to the inhibition of polyphenol oxidases and peroxidases (Drysdale, 1982). Discolouration of calli was evaluated after 30 days. At the higher fusaric acid concentrations (0.25 and 0.3 mM fusaric acid), calli became bleached and greyish rather than brown. This bleaching greyish colour seems to be a characteristic of fusaric acid and was also reported by Drysdale (1984) and Löffler & Mouris (1992). Results are summarised in Figure 9.2.

At fusaric acid concentration of 0.05 and 0.01 mM there was little effect on the relative growth of callus cultures (Figure. 9.1). Addition of fusaric acid at concentration of 0.15 mM gave growth rates of 41.2 % to 48.6 % for the different cultivars. Addition of fusaric acid at concentrations above 0.15 mM to the callus inducing medium significantly affected the growth of the callus (Figure 9.1). At concentrations of 0.25 and 0.3 mM there was very little relative growth and at a concentration of 0.35 mM fusaric acid all cultivars except for 'Late Harvest' had no or even negative relative growth. This may be because 'Late Harvest' is more tolerant to *Fusarium oxysporum* than the other cultivars (Steyn *et al.*, 1991). At 0.4 and 0.5 mM fusaric acid all cultivars had negative relative growth (Figure 9.1). It was apparent that relative growth of callus was inversely related to the toxin concentration. A concentration of 0.2 mM (where 90% of callus died) fusaric acid was chosen and used to select for resistant callus. All calli of the cultivar 'Kimberley Choice' have been inhibited at a concentration of 0.3 mM fusaric acid. This may be due to the fact that 'Kimberley Choice' is the most susceptible cultivar to potato dry rot (Steyn *et al.*, 1991).

5 000 Pieces of callus of each cultivar were transferred to fusaric acid selection medium (containing 0.2 mM fusaric acid) and resistant calli were transferred to fresh medium every 4 weeks for 4 months. After the first week of selection about 90 % of the calli died (Table 9.1), 11.26 % of 'Late Harvest' calli and 6.58 % of 'Kimberley Choice' calli were still viable (Table 9.1). The resistant calli were transferred to fresh medium and then the survival rate was between 93.83% and 86.01% for the different cultivars. During successive transfers to fresh media the survival rate kept increasing (Table 9.1). It was therefore assumed that these calli exhibited resistance to the toxicity of fusaric acid.

Table 9.1 : Response of callus cultures of 5 potato cultivars to fusaric acid (0.2 mM) (LH = Late Harvest; VdP = Vanderplank; KC = Kimberley Choice and UTD = Up-to-Date. Values indicated with the same symbol are not statistically different.

Cultivar	No. of callus cultures initially inoculated	First selection phase		Second selection phase		Third selection phase		Fourth selection phase	
		Number of calli (I)	% (I/A) x 100	Number of calli (II)	% II/I x 100	Number of calli (III)	% III/II x 100	Number of calli (IV)	% IV/III x 100
LH	5000	563a	11,26	518a	92,01	513a	99,03	509a	99,22
KC	5000	329d	6,58	283d	86,01	273d	96,48	268d	98,2
BP1	5000	489c	9,78	434c	88,75	427c	98,39	423c	99,06
VdP	5000	523b	10,46	482b	92,16	480b	99,59	475b	99,0
UTD	5000	486c	9,72	456c	93,83	443c	97,15	440c	99,32
$LSD_T(5\%)$		32		27		24		19	

'Late Harvest' produced significantly more resistant calli than any of the other cultivars, once again explained by the fact that 'Late Harvest' is the most tolerant cultivar. 'Kimberley Choice' produced significantly less resistant calli than the other cultivars. These results correlated with the ranking order Steyn *et al.*, (1991) granted these cultivars.

The resistant calli obtained after selection on the toxic medium were maintained on non-toxic callus initiation medium. Chawla & Wenzel (1987a) found that calli kept continuously on toxic medium became more friable, but regained compactness if kept on non-toxic medium in between. After three months calli were screened on toxic medium again to ascertain the resistance. Of all the calli tested 98,67 % still exhibited resistance to toxicity of fusaric acid, although there was much less relative fresh weight increase on toxic medium (8,7 % to 13,8 %) for the various cultivars than on the non-toxic medium (controls) (156 % - 192 % for various cultivars). There was, however, still more growth than in the unselected material which showed a decrease in relative fresh weight. These results correlated with results found by Chawla & Wenzel (1987a) on barley plants. Parts of actively growing calli were placed on shoot regeneration medium (Chapter 4). Resistant 'Late Harvest' calli produced 137 green plants, 'Vanderplank' 92, 'BP1'

118,'Kimberley Choice' 63, and 'Up-to-Date' 110. Selection on toxic media had an significant inhibitory effect on the regeneration potential of calli (Table 9.2) in comparison with results obtained in Chapter 4. This also correlated with results of other authors (Lynch et al., 1991; Handa *et al.*, 1982).

The leaves of the regenerated plant lines obtained from the selected calli were tested *in vitro* against 0.2 mM fusaric acid. The regeneration potential of regenerated plants with resistance to fusaric acid toxicity was compared to the regeneration potential of the different cultivars obtained in Chapter 4 (Table 9.2). It is apparent that the selected plants showed in some cases (LH 1 - 61; KC 1 - 24; UTD 1 - 60; BP1 1 -47 and VdP 1 - 30) resistance against fusaric acid toxicity whereas some other showed only partial resistance. This variability of regenerated plants in the trait selected for has also been found in rape seed for *Phoma lingam* resistance (Sacristan, 1982), wheat and barley for *Helminthosporium sativum* resistance (Chawla & Wenzel, 1987b) and barley for fusaric acid resistance (Chawla & Wenzel, 1987a). This may be explained by the strong selection pressure which results in different types of mutation acting at different stages of the reaction to a particular toxin or pathogen (Chawla & Wenzel, 1987a). The variability obtained implies furthermore that genetic rather than epigenetic changes at the cellular level have taken place (Chawla & Wenzel, 1987a). This has to be checked and these lines which exhibited an insensitive reaction to the toxin have to be compared with their reaction to the pathogen. These plants will therefore be tested for resistance against *Fusarium oxysporum* in field trials during the next two growing seasons of the potato cultivars. A percentage of 88,3 % of all leaflets taken from regenerated plants of the different cultivars remained green and turgescent during the test in toxin-containing medium whereas 97,6 % of leaflets taken from control plants were bleached and lost their turgor. The leaves of the regenerated plants which survived were also able to produce callus on the suitable medium again.

CONCLUSION

In vitro culture combined with somaclonal variation for the selection of fusaric acid resistant potato phenotypes has potential. Somaclonal variation can already be present

in the explant or can be induced by the technique. Both these methods proved useful for the induction of resistance to fusaric acid in callus and cell suspension cultures. More resistant calli were however obtained when selection pressure (toxin) were added to the medium. This has been demonstrated in a number of crop plants (Wenzel, 1985; Mégnégneu & Branchard, 1988). To reach its full potential it needs a powerful selection system which requires high regeneration capacity in the *in vitro* explant material. The study could allow the use of fusaric acid as a selective agent. Plants resistant to Fusarium wilt have previously been obtained on a fusaric acid containing medium (Shahin & Spivey, 1986). Results indicate that there is selectivity towards calli of the tolerant potato genotypes. Therefore the selection of more tolerant plants could be made between 0.2 and 0.3 mM fusaric acid. The main advantage remains the large number of individuals which can be screened in a small space and the possibility of detecting cell lines with different forms of resistance.

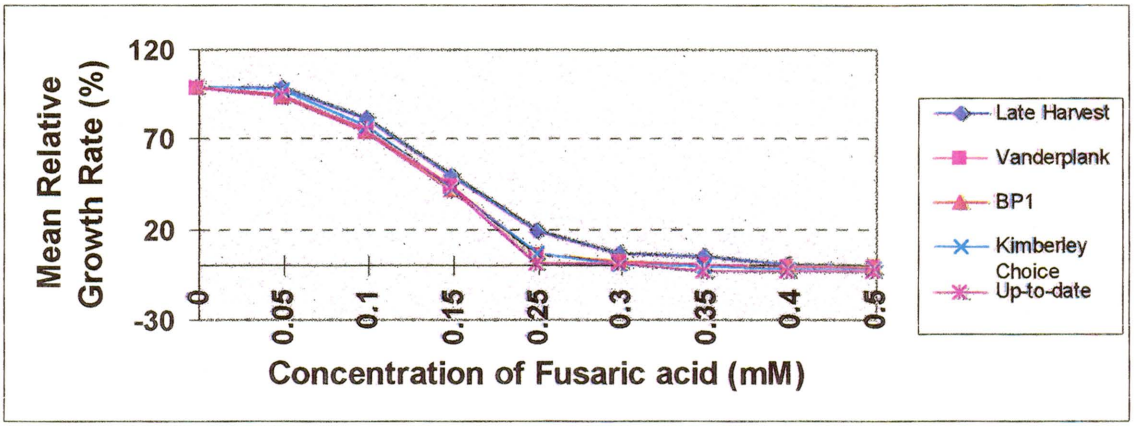

Figure 9.1 Relative growth rates (%) of callus of five potato cultivars at different concentrations (mM) of fusaric acid toxin in callus initiating medium, after 28 days ($P \leq 0.05$, $LSD_T = 3,82$).

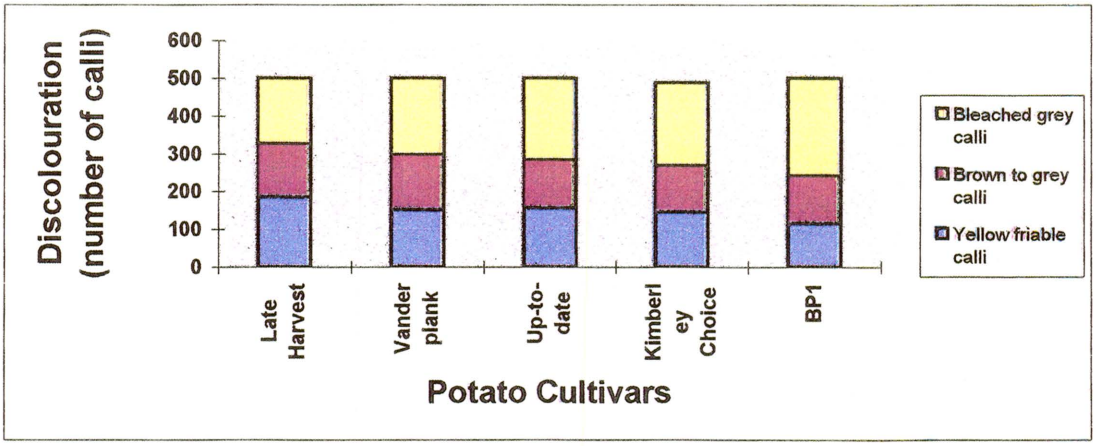

Figure 9.2 The effect/toxicity of fusaric acid on callus cultures of five potato cultivars observed as discolouration of callus. ($P \leq 0.05$, $LSD_T = 28$).

Table 9.2 : Regeneration potential of selected plants with resistance to fusaric acid toxicity compared to unselected plants of 5 potato cultivars ($P \leq 0,05$; $LSD_T = 7,6$) values indicated with the same value are not statistically different

Cultivars	Cultivar clones with resistance to fusaric acid toxicity	Regeneration potential of unselected plants [a]	Regeneration potential of selected calli and cell lines resistant to fusaric acid
Late Harvest (LH)		86,25 % a	
	LH 1 - 61		84,3 - 86,12 % a
	LH 62 - 119		68,9 - 72,3% b
	LH 120 - 137		50,3 - 57,2% c
K C[b]		67,5% b	
	KC 1 - 42		59,7 - 62,6% c
	KC 25 - 32		40,2 - 46,3% d
	KC 33 - 63		< 20% f
UTD[c]		63,75% bc	
	UTD 1 - 60		58,2 - 59,9% c
	UTD 61 - 90		42,1 - 44,3% d
	UTD 91 - 110		< 23,6% f
BP 1		52,7% c	
	BP 1 1 - 47		46,7 - 48,3% d
	BP 1 47 - 118		< 32,3% e
VdP[d]		52,5% c	
	VdP 1 - 30		42,6 - 44,2% d
	VdP 31 - 92		< 30,6% e

[a] Obtained in Chapter 4. Regeneration potential refers to the percentage of explants which produced shoot on shoot induction media. [b]KC = Kimberley Choice
[c]UTD = Up-to-Date; [d]VdP = Vanderplank

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10. EFFECT OF FUNGAL CULTURE FILTRATE OF *FUSARIUM OXYSPORUM* ON CELL SUSPENSION AND CALLUS CULTURES OF POTATO (*SOLANUM TUBEROSUM* L.) IN SOUTH AFRICA

INTRODUCTION

Fusarium oxysporum causes wilt, stem-end-rot and dry rot of potatoes in South Africa. It is a soilborne pathogen and the only effective control being the use of resistant varieties. Currently there is no potato cultivar in South Africa which is resistant to *Fusarium oxysporum* causing dry rot. Plant tissue cultures could be used to either select disease resistant genotypes by *in vitro* selection or to evaluate disease resistance. A protocol that has been widely used for the selection of disease resistant lines is to grow callus in the presence of a culture filtrate or toxins (Mégnégneau & Branchard, 1991). Although this method does not assure that plants regenerated from resistant calli will also be resistant to the pathogen, it has been effectively applied to several plant-pathogen systems (Behnke, 1979, 1980; Sacristan, 1982; Thanutong *et al.*, 1983; Hartman *et al.*, 1984; Toyoda et al., 1984; Arcioni *et al.*, 1987; Chawla & Wenzel, 1987).

The use of tissue culture for the evaluation of disease resistance or to obtain plants with improved resistance is dependent on a positive correlation between *in vitro* culture filtrate resistance and whole plant resistance. Gray *et al.*, (1986), Willmot *et al.*, (1989) and Connel *et al.*, (1990) developed a tissue culture protocol to differentiate between calli derived from resistant or susceptible cultivars to the pathogen, by adding fungal culture filtrate containing phytotoxic metabolites to a callus growth medium. The extent of inhibition of callus growth caused by culture filtrate treatment was related to the level of resistance of the cultivar. *Fusarium oxysporum* is an appropriate pathogen because of its high capacity for synthesizing toxins *in vitro*. *In vitro* selection of mutants among populations of cultured plant cells offers a novel source of genetic variability to plant breeders (Shahin & Spivey, 1986). Novel disease-resistant mutants of a small number of crop species have been recovered from cells selected in culture for their resistance to phytotoxin (Wenzel, 1985).

A correlation exists between fusaric acid production of *Fusarium oxysporum* and the development of dry rot in potatoes in South Africa (Venter *et al.*, 1996). The first *in vitro* selection was performed using callus from leaf discs as described in Chapter 9. Fusaric acid, a non-host specific toxin, was used as a selection agent. Callus of various potato cultivars were subjected to the toxin and the surviving cell colonies were subjected to further toxin. The toxin-resistant calli were then induced to form shoots on toxin-free regeneration medium. Calli on regeneration medium usually produced fluffy white areas with green domes prior to shoot appearance. Plants were recovered from these cultures and exhibited resistance to fusaric acid. The objective of this paper is to describe the effect of culture filtrates of *Fusarium oxysporum* on cell suspension and callus culture of potato cultivars and by doing so develop an *in vitro* evaluation method by which potato cultivars with improved resistance to *Fusarium oxysporum* could be selected.

MATERIALS AND METHODS

Plant material. Virus-free potato material of five cultivars, 'Late Harvest', 'BP1', 'Up-to-Date', 'Kimberley Choice' and 'Vanderplank', was obtained from the genebank at the ARC-Roodeplaat Vegetable and Ornamental Plant Institute. *In vitro* culture of cultivars and conditions were used as described previously (Chapters 2,3,4 and 5).

***Fusarium oxysporum* isolates**. A highly aggressive isolate of *Fusarium oxysporum* was kindly supplied by D.J. Theron from the ARC-Roodeplaat Vegetable and Ornamental Plant Institute. The isolate was isolated from potato plant material with dry-rot symptoms and the virulence ascertained (Venter *et al.*, 1996).

Culture filtrate. *Fusarium oxysporum* isolates were cultured on potato dextrose agar (Difco) in 90 x 90 mm petri dishes. Cultures were maintained as described by Venter et al., (1992). Two agar blocks ($1mm^3$) of 10 day old cultures were placed in 100 mℓ of Fusarium culture medium and the liquid cultures were incubated (Venter & Steyn, 1997). Filtrates were collected by vacuum filtration (Whatman #41), filter sterilized with a 0.22 µm Millipore filter unit and stored at -20 °C to -70 °C until use. The pH of the crude filtrate was adjusted to pH 5.7 with 1N HCl. To avoid thermal degradation of toxic compounds in the fungal culture filtrate, it was added to luque

warm autoclaved medium. Preliminary tests of culture filtrate toxicity involved dilution series studies. The different proportions used in this study were 4, 6, 8 and 10% (v/v). Controls contained the liquid-fungal-growth medium.

Cell suspension cultures : Well established cell suspension cultures were obtained as described previously (Chapter 3). About 7 days before plating the cells, the Fusarium culture filtrates were added to the cell suspension cultures. The cell suspension cultures were plated on callus initiating medium (Chapter 3) containing the culture filtrates at the same concentrations as in the cell suspension medium. Fifty petri dishes (9 cm diameter) were plated per treatment. At the same time cell suspension cultures which had not been inoculated with culture filtrate were also plated on the callus initiation medium containing different concentrations of culture filtrate. Microcalli that were formed were subjected to shoot induction medium (Chapter 4) and the regenerated shoots were rooted (Chapter 4) and the regenerated plants were screened for resistance to the culture filtrate as described in Chapter 9. Dose response curves of the cells to the culture filtrate of *Fusarium oxysporum* were determined to find the concentration where 90% of the cells were killed. Relative fresh mass increase was determined according to the following equation :

$$\frac{Xn - Xo}{Xc - Xo} \times 100$$

where Xn = fresh mass for concentration n
Xc = fresh mass for the control
Xo = initial fresh mass

Analysis of fusaric acid in culture filtrates : Culture filtrates from 30-day old cultures of *Fusarium oxysporum* were tested for the presence of fusaric acid using Reversed Phase HPLC (High Performance Liquid Chromatography) as described previously (Venter & Steyn, 1997). The effect of 6% culture filtrate on the callus cultures was determined over a period of time and correlated with increase of fusaric acid in culture filtrates determined by HPLC. Culture filtrates were sampled every second day for 20 days and media prepared as described above.

RESULTS AND DISCUSSION

Dose responses of the cell suspension cultures of the five potato cultivars are shown in Figure 10.1. The results indicated that 8% of the culture filtrate was sufficient to kill all the original cells of all the cultivars except for 'Late Harvest'. 'Late Harvest' cells were killed at a 10% culture filtrate concentration. At all concentrations 'Late Harvest' showed a significant better growth rate than that of 'Vanderplank', 'Up-to-Date' and 'BP1', whereas 'Kimberley Choice' had a significant poorer growth rate than all the other cultivars ($P < 0{,}001$). This may be explained by the fact that 'Late Harvest' is the most tolerant cultivar to *F. oxysporum* causing dry rot while 'BP1', 'Up-to-Date' and 'Vanderplank' are considered to be moderately resistant and 'Kimberley Choice' the most susceptible cultivar (Steyn *et al.*, 1991). At a concentration of 8% culture filtrate 90% of the potato cells died. (Figure 10.1).

Resistant plants were recovered by three routes, namely (1) without selection pressure, (2) with selection against Fusarium culture filtrate only during the callus phase and (3) selection against Fusarium culture filtrate during cell suspension as well as callus phases (Table 10.1) all the cultivars produced less microcalli, shoots, plantlets and resistant plants on media where only the callus initiating medium contained the culture filtrate compared to where both the cell suspension culture as well as the callus initiating medium contained culture filtrate. Cell suspension and callus cultures with no selection pressure produced by far less resistant individuals than the other two treatments, although some resistant individuals were obtained. 'Late Harvest' produced more resistant individuals than any other cultivar and 'Kimberley Choice' less than all the other. There was not much variation between the other three cultivars. This supported the ranking order Steyn *et al.*, (1991) granted these cultivars.

In greenhouse tests 74% of the regenerated plants were phenotypically similar to the original cultivars and although a lack of obvious morphological abnormalities is shown, it is too early to draw conclusions about their usefulness. Similar results were obtained by Shahin & Spivey (1986) on greenhouse trials with tomato plants resistant to *Fusarium oxysporum* f. sp. *lycopersici* race 3. The other 26% plants varied slightly in some of the morphological characteristics, such as height, leaf shape, tuber shape and flower characteristics.

A total of 436 'Late Harvest', 219 'Vanderplank', 259 'BP1', 264 'Up-to-Date' and 132 'Kimberley Choice' individuals exhibiting resistance to *Fusarium oxysporum* culture filtrate were obtained.

The concentration of fusaric acid in the culture filtrates was determined by HPLC (Venter, *et al., 1996;* Venter & Steyn, 1997). The concentration amounted to approximately 2.8 mM after 28 days. This concentration is higher than the 1 mM after 30 days found by Löffler & Mouris (1992), and the *circa* 2 mM after 35 days found by Matsui & Watanabe (1988), but much lower than that mentioned by Dobson *et al.*, (1967), (*circa* 7 mM after 7 days). Time has an influence on the effect the culture filtrate has on the callus cultures (Figure 10.2). The accumulation of fusaric acid in the medium coincides with the increase of toxicity as found in the assay. After 6 days fusaric acid was found in the culture filtrates and the concentration increases in time (Venter & Steyn, 1997). According to the colour bioassay, toxic activity started at day 8 and at day 18 almost all the calli of all the cultivars were completely greyish which is a characteristic symptom for the presence of fusaric acid.

More than one component could be responsible for the observed toxic activity of the culture filtrate. However, according to results obtained, the production of fusaric acid coincides with the increase of toxic activity in the culture filtrate and the production of fusaric acid in culture filtrates (Venter & Steyn, 1997, Venter, et al., 1996). Moreover, the symptoms observed after exposure of the callus to culture filtrate were typical for the presence of fusaric acid (Drysdale, 1982; Löffler & Mouris, 1992 and results obtained in Chapter 9. Therefore these symptoms seem to be at least partially due to fusaric acid. It is also shown in this study as well as before (Venter *et al.*, 1996) that fusaric acid is produced by *Fusarium oxysporum* causing dry rot of potatoes. This compound is toxic to both differentiated as well as non-differentiated tissues of potato and it was also found that fusaric acid plays an important role in potato dry rot development of various cultivars (Venter *et al.*, 1996). Regenerated plants should, however, be tested in field trials for resistance against the pathogen *Fusarium oxysporum*. It is possible that the approaches described here will have an impact on the production of novel disease-resistant potato plants, particularly when conventional breeding methods have failed to provide an answer to a specific disease problem. The extent to which it can be exploited will, however, depend on whether the recovered

undesirable heritable traits could impede the breeding process. It is clear from our results that undesirable traits were also recovered besides the targeted one. Unless there is a method to sort out those undesirable changes, the use of *in vitro* selection will be cumbersome to the plant breeder. Nevertheless, *in vitro* selection has its advantage as a potential source of novel breeding material for traits otherwise unobtainable by conventional breeding methods (Shahin & Spivey, 1987).

CONCLUSION

It is shown in this study that fusaric acid is produced by *Fusarium oxysporium* causing dry rot of potatoes. Regenerated plants exhibiting *in vitro* resistance against the culture filtrate of *F. oxysporum* should be tested in field trials for resistance against the pathogen *F. oxysporum.* Results obtained in this study warns that applying *in vitro* selection for the improved resistance of potato cultivars to *Fusarium oxysporum*, by adding only one characteristic without altering the remaining genotype should be researched carefully. It can be anticipated that *in vitro* selection for disease resistance as a breeding tool will continue to expand in future.

Table 10.1 : Summary of *in vitro* selection of Fusarium resistant plants from cell suspension cultures of five potato cultivars

Treatment[a]		Microcalli [b]					Microcalli with shoots ©					Total number of plantlets					Number of resistant individuals [d]				
Cell suspension medium	Callus initiating medium	LH	VdP	BP1	KC	UTD	LH	VdP	BP1	KC	UTD	LH	VdP	BP1	KC	UTD	LH	VdP	BP1	KC	UTD
0 CF	0 CF	3012	1816	2481	2316	2126	452	272	372	347	319	3706	1414	2120	1804	1818	32	23	21	8	19
4% CF	4% CF	2216	1056	1446	933	1252	288	137	188	121	183	1612	712	790	435	897	193	85	94	52	107
6% CF	6% CF	1195	307	429	148	309	143	37	52	18	40	944	192	218	65	196	113	23	26	7	24
8% CF	8% CF	204	0	0	0	0	24	0	0	0	0	158	0	0	0	0	19	0	0	0	0
10% CF	10% CF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0 CF	4% CF	2182	1031	1281	916	1018	327	155	192	137	163	2158	604	806	493	799	259	72	96	59	96
0 CF	6% CF	1016	218	328	101	168	132	28	43	13	31	871	109	181	47	143	104	16	22	6	18
0 CF	8% CF	169	0	0	0	0	20	0	0	0	0	132	0	0	0	0	16	0	0	0	0
0 CF	10% CF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] *Fusarium oxysporum* culture filtrate (CF).

[b] The number indicates those Microcalli derived from cell colonies that survived selection pressure as indicated by the treatment.

© Number of Microcalli that produced shoots on shoot initiating medium (Chapter 4) without culture filtrates.

[d] Resistant individuals tested according to method described by Venter, Chapter 11 (callus selection).

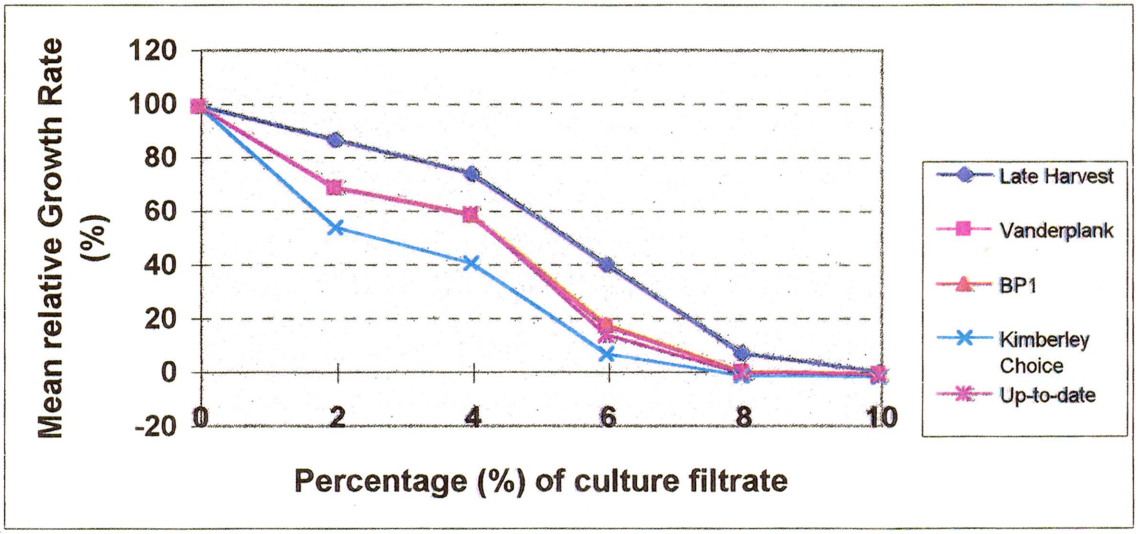

Figure 10.1 Dose response curves of cell suspension cultures of five potato cultivars to a culture filtrate of *Fusarium oxysporum*. ($P \leq 0.05$, $LSD_T = 11,76$).

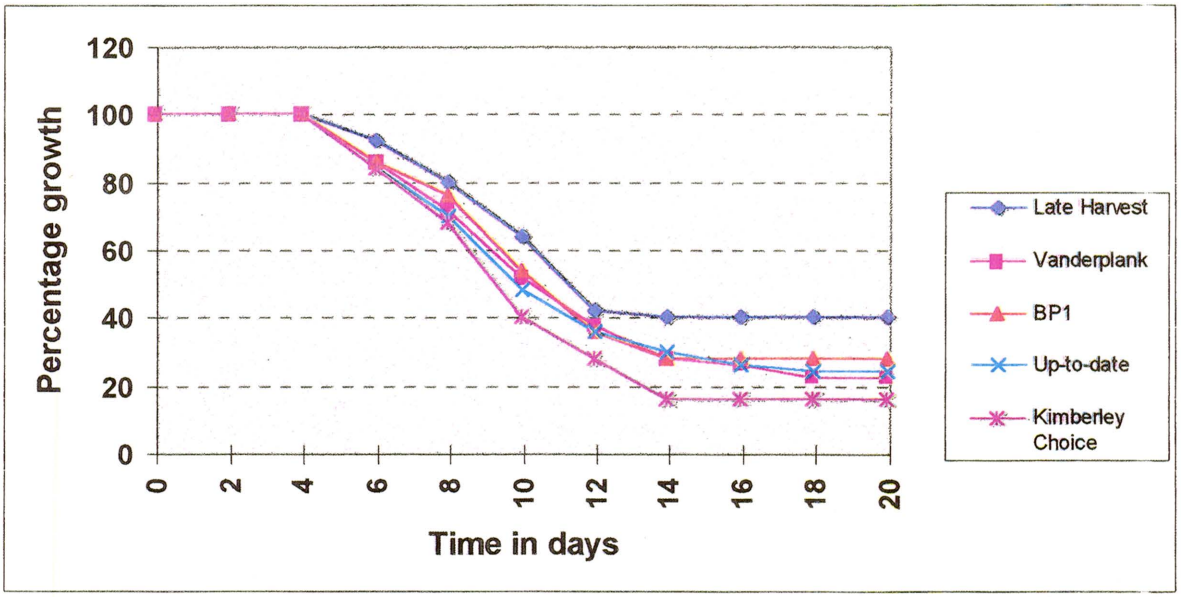

Figure 10.2 The influence of time on the effect of *Fusarium oxysporum* culture filtrate on callus cultures of five potato cultivars (% Growth = $^{x}/50 \times 100$ calli is alive), ($P \leq 0.05$, $LSD_T = 4,8$).

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11. A RAPID VIABILITY ASSAY FOR GROWTH RESPONSE OF POTATO CELL SUSPENSION AND CALLUS CULTURES TO FUSARIC ACID AND CULTURE FILTRATE OF *FUSARIUM OXYSPORUM*

INTRODUCTION

The utilization of cell cultures for the selection of desirable genotypes can be a valuable tool for the breeder, but it is still handicapped by technical problems (Wenzel, 1985). Selection for resistance using inhibitors or toxins requires precise definition of the exposure parameters of the cell culture. The factor used for the selection must be tested over a wide range of concentrations in order to determine the growth response of the cell populations. Cell sensitivity can be determined by several methods such as cell counting, fresh and dry mass, packed cell volume, mitotic index and viability staining. Some of these methods are labourious and cumbersome, since they require large quantities of cells, several repetitions and tedious microscopic examinations (Tepper *et al.*, 1991). The measurements are often very variable and may require sophisticated and expensive equipment. In many cases the sampling and measuring are not frequent enough and may result in erroneous conclusions (Gonzales & Widholm, 1985). Viable cells of a variety of organisms are able to reduce various tetrazolium salts (Smith, 1951). Tetrazolium salts are reduced to red formazaan (Nachlas *et al.*, 1960, Towill & Mazur, 1975). The reduction occurs in the mitochondria by the tetrazolium accepting electrons from the electron-transport chain (Nachlas *et al.*, 1960). Steponkus & Lanphear (1967) reported an assay in which the red formazaan formed by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by viable cells is extracted with ethanol and its absorbance is measured. Towill & Mazur (1975) reported further studies on the reduction of TTC as a viability assay for plant tissue cultures.

TTC reduction has been used as a viability assay for many years (Bennet & Loomis, 1949) and represents a simple rapid means of assessing viability when dealing with large numbers of samples. Towill & Mazur (1975) have shown a correlation between the amount of formazan formed by a sample and the percentage of viable cells within the sample as measured by the criterion of cell division. Steponkus (1971) has also concluded that the TTC procedure gives a reasonably

accurate estimate of viability for leaf discs, stem sections and tissue cultures. Correspondence between regrowth and TTC reduction is not always absolute (Steponkus & Lanphear, 1967), therefore initial studies using two or more methods of determining viability are advisable. The rapidity of the test and the correspondence between the two assays in defining the largest decrease in survival demonstrated that the TTC assay is useful for many viability studies. This paper presents a simple, rapid growth measuring method developed to determine the *in vitro* sensitivity of potato cells to fusaric acid and a culture filtrate of *Fusarium oxysporum* and established a selection system for cell lines of potato resistant to the toxins produced by *F. oxysporum.*

Materials and Methods

***Fusarium oxysporum* isolates :** A highly aggressive isolate of *Fusarium oxysporum* was kindly supplied by D.J.. Theron from the ARC-Roodeplaat Vegetable and Ornamental Plant Institute. The isolate was isolated from potato plant material with dry rot symptoms and the virulence ascertained (Venter *et al.*, 1996).

Culture filtrate : Culture filtrates and toxic media were prepared as described in chapters 9 and 10. Media contained 4, 5, 6, 7, 8, 10 and 12% (v/v) culture filtrates of *Fusarium oxysporum* causing dry rot on potatoes.

Fusaric acid : Fusaric acid containing media were prepared as described in chapter 9 at concentrations of 0.0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 1.0 and 1.5mM fusaric acid.

Cell suspension cultures : Cell suspension cultures of the potato cultivars 'Late Harvest', 'BP1', 'Up-to-Date', 'Kimberley Choice' and 'Vanderplank' were prepared as described in chapter 3.

Cell viability assay

The TTC was dissolved in the reaction mixture. The reaction mixture was prepared by mixing

(v/v) (2:1) 0.05M sodium phosphate buffer pH (7.5) and liquid MS medium (Murashige & Skoog, 1962). Two cm^3 cells were subcultured to sterile centrifuge tubes and cells were centrifuged at 200 x g (1 000 r.p.m.) for 7 min. Packed cell volume was recorded and supernatant discarded. The TTC solution (5 cm^3) was added to the remaining cells and incubated at 26± 1°C in the dark for 18 - 22h. After incubation, the cells were pelleted by centrifugation at 1 000 r.p.m. for 7 min and the supernatant discarded.

The cells were washed once with sterile distilled water, centrifuged again and the supernatant discarded. The red formazaan was extracted from the pelleted cells with 5 cm^3 of 96% ethanol for 30 min (of which 15 min at 60 °C in the beginning). This brief heating aided in extracting the formazaan from large clumps. The absorbance of the extract was read at 485 nm with a Beckman spectrophotometer. Optimum TTC concentration, pH and incubation time have been determined before. Optimum TTC concentration was 0.2%, optimum pH 7.5 and incubation time 18 h.

Resistance of leaf tissue of the regenerated plantlets to the culture filtrate and fusaric acid was tested by immersing leaf segments of plantlets *in vitro* in the liquid subculture medium with the culture filtrate or the toxin (0.2 mM fusaric acid) as described in Chapter 9.

Growth rate and viability of cell suspension cultures and calli on the toxic medium were measured to determine the toxicity of the culture filtrates and fusaric acid. Growth rate was calculated as described in Chapter 9 and 10 and the TTC assay as described above was used as a viability assay for the cell suspension and callus cultures exposed to the toxic media.

13.3 Results and Discussion

The cell suspension cultures as well as the callus cultures grew well on the subculture media and their growth were not affected by fungal culture filtrate media up to 4% and fusaric acid media up to 0,1 mM. However, the cell suspension cultures became bleached and greyish on subculture media containing 8 - 12% fungal culture filtrate and 0.35 to 1.5 mM fusaric acid. From results in Figures 11.1 and 11.2 it is obvious that 'Late Harvest' cell suspension cultures were more tolerant to both the fusaric acid as well as culture filtrate containing media than any of the other

cultivars, whereas 'Kimberley Choice' was more susceptible (≤0,5). These results correlated with results obtained in previous studies (Chapter 9, 10) as well as with the ranking order Steyn *et al.*, (1991) granted the potato cultivars.

A correlation was found between the three methods evaluated. A lag phase of about two days and a fast growth rate between the second and fifth days for the various potato cultivars was observed (Figures 11.3 and 11.4). Figures 11.3 and 11. 4 represent the growth curves of the cells. At low fusaric acid (0.01 and 0.02 mM) and culture filtrate concentrations the cells continued to grow until about the 20th day. The viability assays matched the percentage growth and packed cell volume curves. It can be seen that the difference in absorbance (TTC assay) between lethal and non-lethal fusaric acid and culture filtrate concentrations is obvious, although 0.03 mM fusaric acid and 5% culture filtrate showed an intermediate response. This was also observed by Jullien(1988) and Tepper *et al.*, 1991). Similar curves were obtained by Jullien (1988) although he worked with mesophyll cells of *Asparagus officinalis* and counted cell populations under a microscope in order to determine the growth rate and viability. Tepper *et al.*, (1991) also obtained similar results even though they worked with suspension cultures of *Asparagus officinalis* and used packed cell volume to determine growth rate and a photometric system to determine the viability. The TTC method makes it possible to follow continuously the response of cultured cells to fusaric acid and culture filtrates of *F. oxysporum.* The advantages of this approach are that small quantities of plant cells in suspension are sufficient for several replications and treatments, frequent measurements can be taken since the time required for each determination is short and simple, the same system can be used to measure the effect of various other factors on cell growth and development *in vitro.* Another advantage is that the type of growth and texture of aggregates do not influence results which is so often a problem when using other methods such as the photometric system described by Tepper *et al.*, (1991).

Figures 11.1 and 11.2 show the effect of fusaric acid and culture filtrates on the growth of the cell suspension cultures. All cell cultures of 'Kimberley Choice' died on subculture medium containing 0.3 mM fusaric acid and 7% culture filtrate, whereas cell cultures of 'Vanderplank', 'Up-to-Date' and 'BP1' died on subculture medium containing 0.4 mM fusaric acid and 10% culture filtrate. Cell cultures of 'Late Harvest' died at 0.5 mM fusaric acid and 12% culture.

Cell suspension cultures were plated on toxic media containing the same concentrations of fusaric acid and culture filtrate than that of the cell suspension media. All callus pieces of 'Kimberley Choice' died on media containing 0.3 mM fusaric acid and 7% culture filtrate as was the case with the cell suspension cultures. The other cultivars showed the same pattern. Many of the callus pieces of the other cultivars also died on the medium containing 0.3 mM fusaric acid, however, depending on the cultivar, 10 - 20% of the callus pieces survived and grew well. Callus of 'Late Harvest' was less affected by 0.4 mM fusaric acid and 8% culture filtrate than the other cultivars, but 40 - 60% of the callus pieces died on this medium. One mM fusaric acid killed pieces of all the cultivars. These results once again correlated with the ranking order Steyn *et al.*, (1991) granted the cultivars and it can be said that the resistance of the potato cell suspension cultures and calli was correlated with the resistance of the original plants to Fusarium dry rot caused by the fungus. This fact suggests that bioassays of potato calli and cell suspension cultures with culture filtrates and fusaric acid are good assay systems for estimating the resistance of potato plants to the disease, because the influence of any other pathogen is eliminated.

This system can therefore be used for *in vitro* selection of resistant cell lines of potatoes to Fusarium dry rot as reported for other plant species (Gengenbach & Green, 1975; Behnke, 1980a,b; Hartman *et al.*, 1984; Arai & Takeuchi, 1993). The effects of the culture filtrates and fusaric acid on leaves also agree with pathogenicities of the fungi to original plants. This implies that culture filtrates of the pathogens can be used for the estimation of resistance to the disease caused by the fungi. Fusaric acid, which is said to be a non-specific toxin produced by *Fusarium*, has almost the same effect than that of the culture filtrates on the growth and viability of cells and calli - especially at low concentrations. At concentrations higher than 0.45 mM the difference in response of the calli between the cultivars is not clear. In the present study a system for estimating the resistance of potato cultivars to Fusarium dry rot was established and it will therefore be possible to produce new cultivars or more tolerant cultivars from a selected mutant cell. This correlates with results obtained by Arai & Takeuchi (1993), Gray *et al.*, (1986), Arai *et al.*, (1989) & Simard *et al.*, (1992) although obtained with other species.

CONCLUSION

The system developed can be a handy tool for breeding of potato cultivars resistant to Fusarium dry rot. This system is, however, dependent on linkage to the conventional potato breeding programme. The linkage will ensure that the tissue culture material developed has the agronomic performance that is essential.

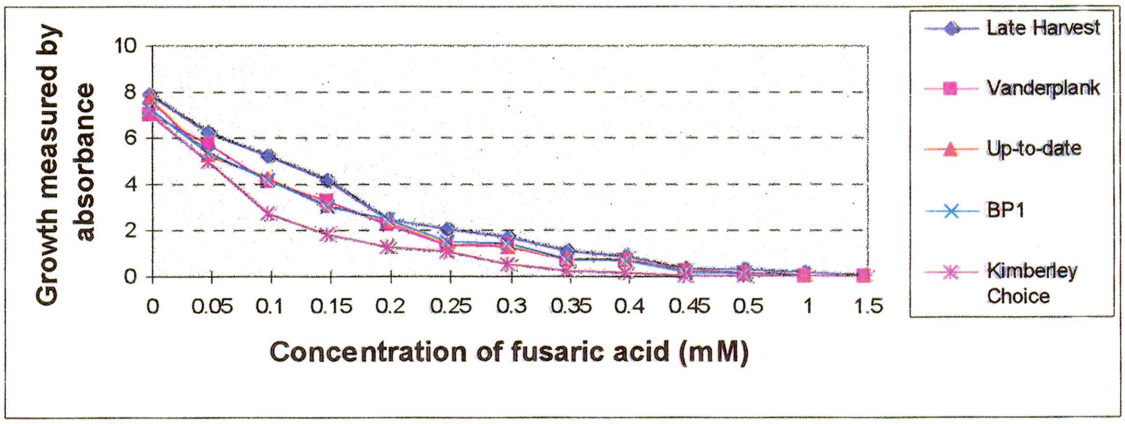

Figure 11.1A Dose response curve for the effect of fusaric acid on the growth of cell suspension cultures of five potato cultivars determined by TTC , ($P \leq 0.05$, $LSD_T = 0,89$).

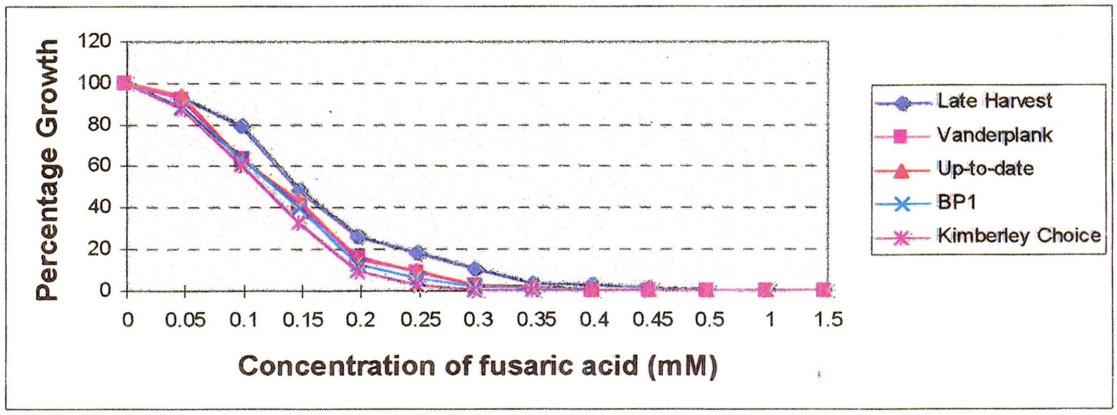

Figure 11.1B Dose response curve for the effect of fusaric acid on cell suspension cultures of five potato cultivars determined by percentage growth , ($P \leq 0.05$, $LSD_T = 8,6$).

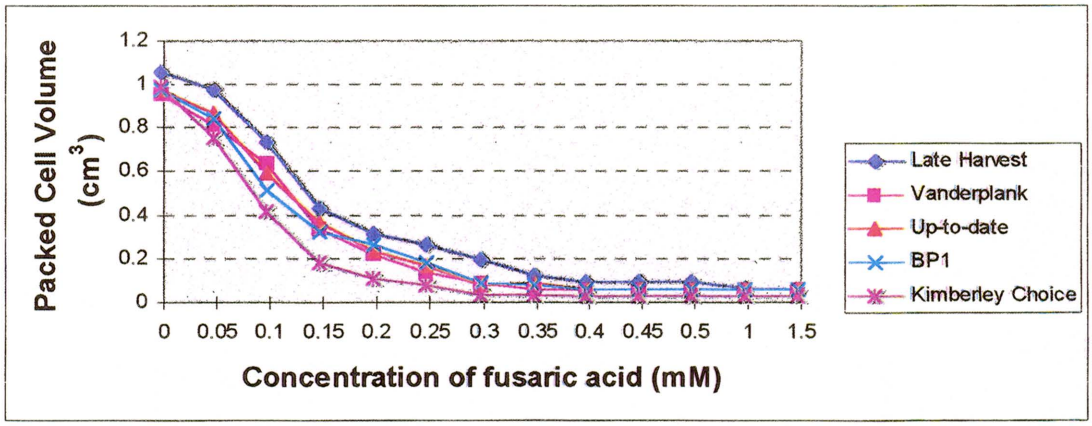

Figure 11.1C Dose response curve for the effect of fusaric acid on cell suspension cultures of five potato cultivars determined by packed cell volume , ($P \leq 0.05$, $LSD_T = 0,087$).

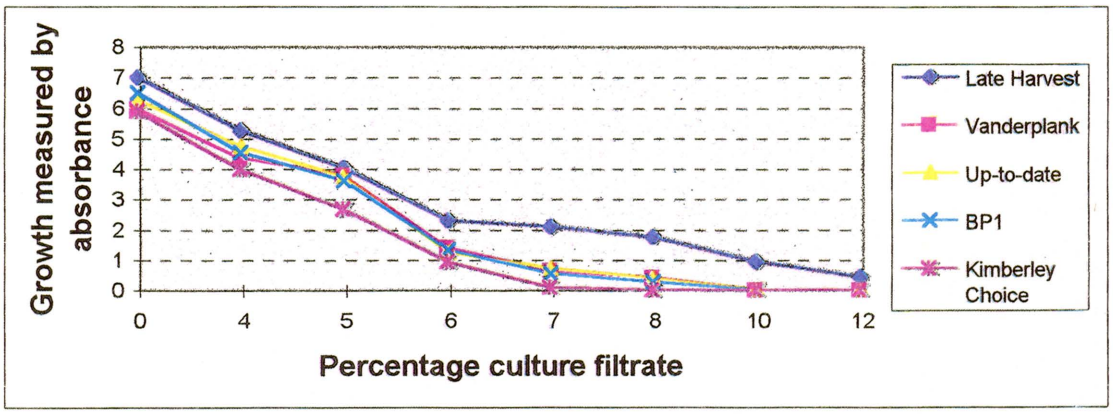

Figure 11.2A Dose response curve for the effect of *Fusarium oxysporum* culture filtrates on the growth of cell suspension cultures of five potato cultivars determined by TTC , ($P \leq 0.05$, $LSD_T = 0,96$).

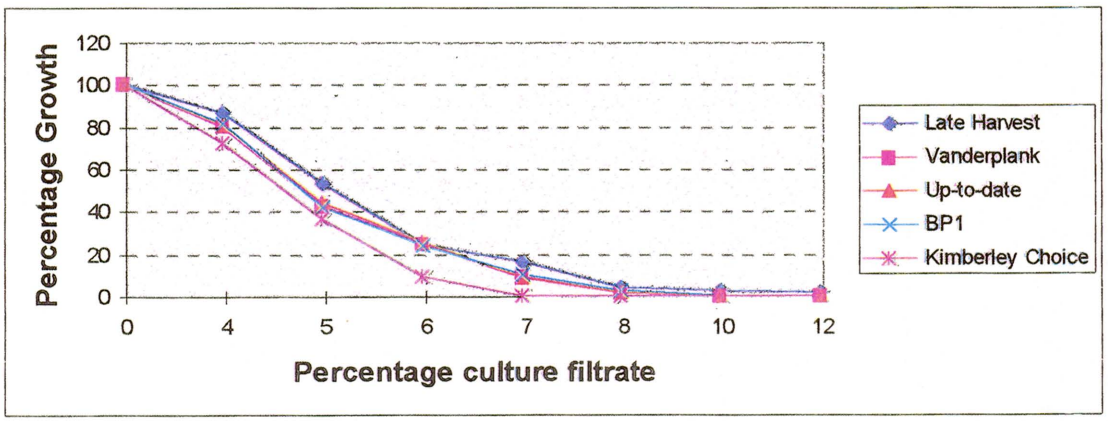

Figure 11.2B Dose response curve for the effect of *Fusarium oxysporum* culture filtrates in cell suspension cultures of five potato cultivars determined by percentage growth , ($P \leq 0.05$, $LSD_T = 9,24$).

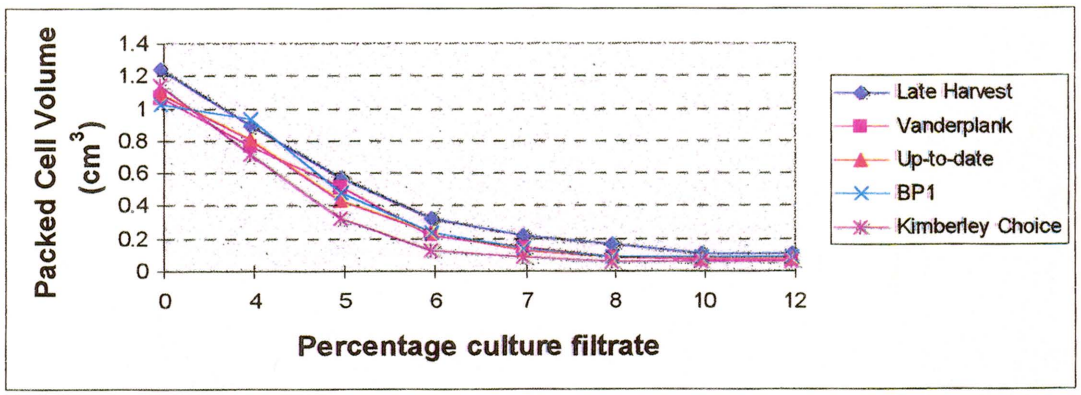

Figure 11.2C Dose response curve for the effect of *Fusarium oxysporum* culture filtrates in cell suspension cultures of five potato cultivars determined by packed cell volume , ($P \leq 0.05$, $LSD_T = 0,093$).

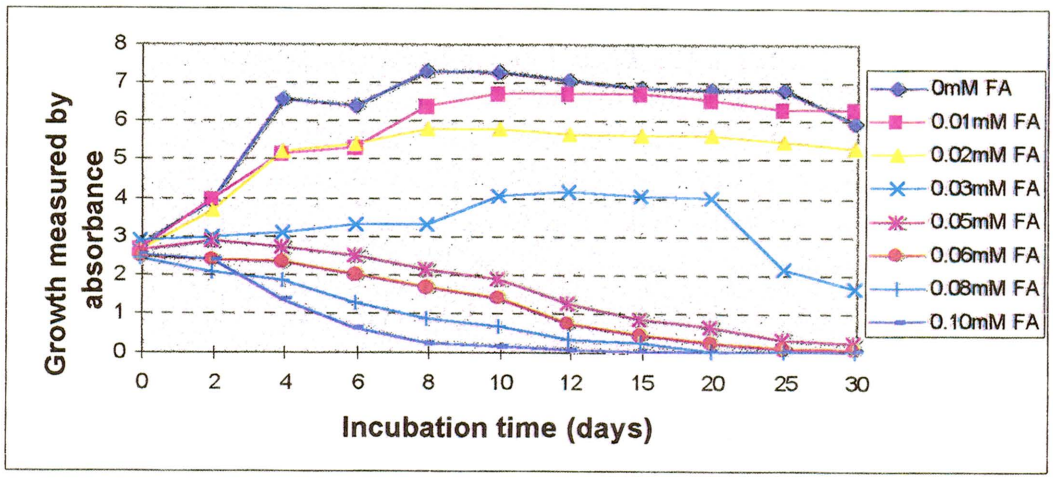

Figure 11.3A The influence of time and concentration of fusaric acid on growth of callus cultures of five potato cultivars determined by TTC , ($P \leq 0.05$, $LSD_T = 1,26$).

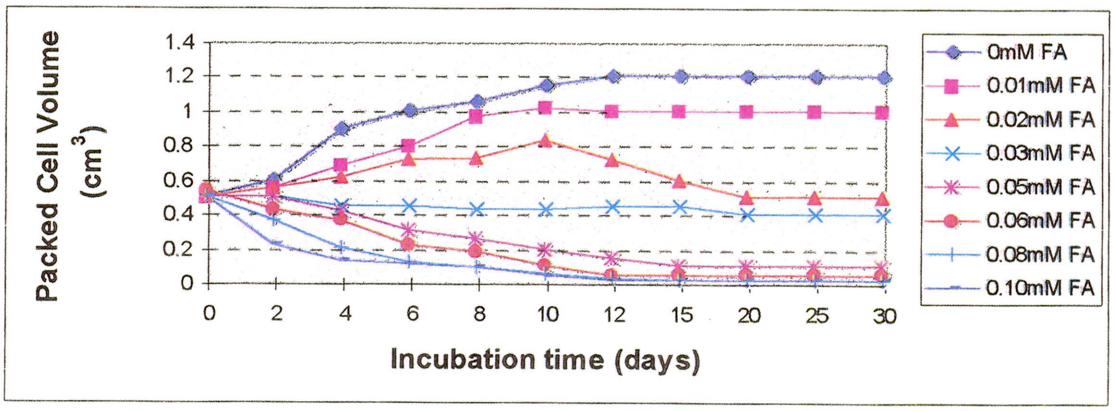

Figure 11.3B The influence of time and concentration of fusaric acid on the growth of callus cultures of five potato cultivars determined by Packed Cell Volume , ($P \leq 0.05$, $LSD_T = 0,14$).

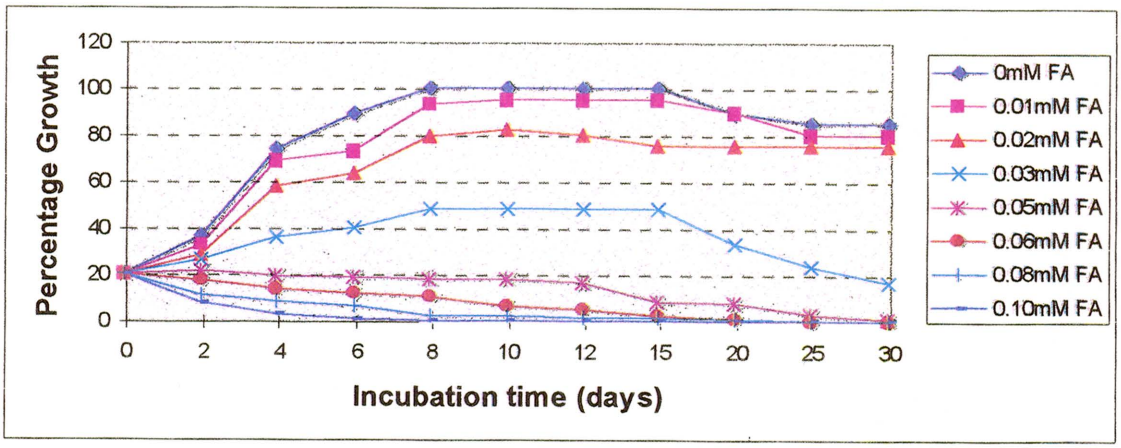

Figure 11.3C The influence of time and concentration of fusaric acid on the growth of callus cultures of five potato cultivars determined by Percentage growth (% Growth = $^{x}/50$ x 100 calli is alive) , ($P \leq 0.05$, $LSD_T = 6,82$).

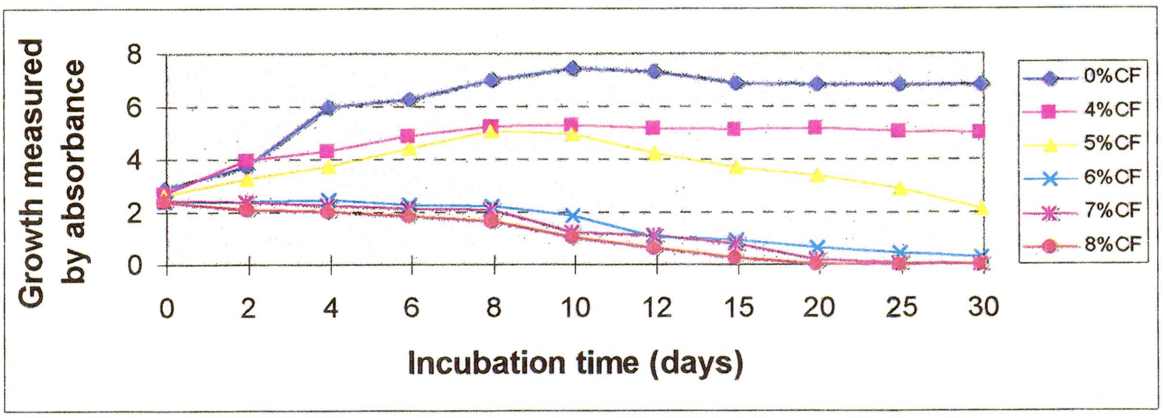

Figure 11.4A The influence of time and concentration of *Fusarium oxysporum* culture filtrate on growth of callus cultures of five potato cultivars determined by TTC , ($P \leq 0.05$, $LSD_T = 1,22$).

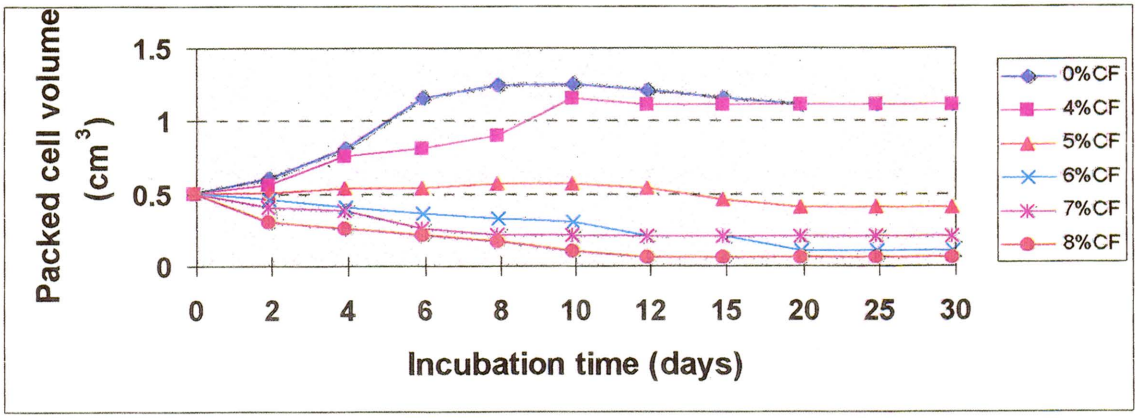

Figure 11.4B The influence of time and concentration of *Fusarium oxysporum* culture filtrate on growth of callus cultures of five potato cultivars determined by Packed Cell Volume , ($P \leq 0.05$, $LSD_T = 0,54$).

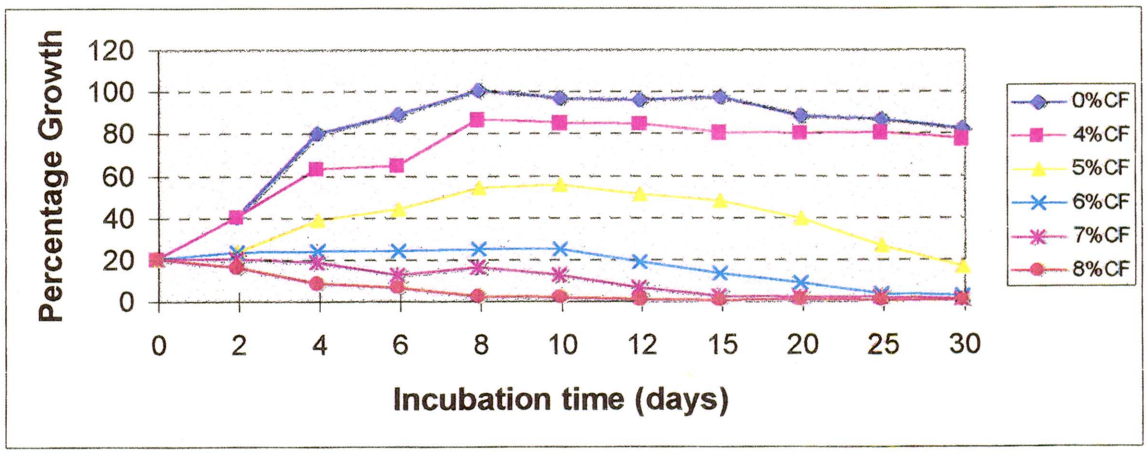

Figure 11.4C The influence of time and concentration of *Fusarium oxysporum* culture filtrate on growth of callus cultures of five potato cultivars determined by percentage growth. , ($P \leq 0.05$, $LSD_T = 9,68$).

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12. FIELD EVALUATION OF LATE HARVEST CLONES WITH IMPROVED *IN VITRO* RESISTANCE AGAINST FUSARIC ACID AND/OR CULTURE FILTRATE OF *FUSARIUM OXYSPORUM*

INTRODUCTION

Fusarium oxysporum W.C. Snyder & Hans, causes dry rot, stem-end rot and wilt of potatoes (*Solanum tuberosum*) (Nelson *et al.*, 1981). On a potato breeding Indaba held in 1991 at the ARC-Roodeplaat, the potato breeders and pathologists felt that screening for Fusarium resistance is important and that an effective screening method is essential. It was stated that cultivars, clones or breeding lines with improved *Fusarium* resistance were needed, but these improved cultivars and/or breeding lines should not differ from their breeding parents in other characteristics. Conventional breeding strategies are time-consuming and until recently the Fusarium resistance breeding programme was based on conventional breeding methods. Alternative methods for the development and induction of new characteristics in cultivars and clones should be investigated. Plant tissue culture has in recent years attracted growing interest form plant pathologists and breeders aiming for disease resistance, since it could meet the requirements for screening a large number of individuals in a small space (Chawla & Wenzel, 1987). At the meeting it was decided that an effective *in vitro* screening and selection model needed to be developed for South African potato cultivars. Several studies were conducted with the aim to develop such a model (Chapters 9 to 11). The aim of this study was the field evaluation of clones of the potato cultivar 'Late Harvest' with improved *in vitro* resistance to fusaric acid and *F. oxysporum* culture filtrates which was induced using *in vitro* screening and selection techniques described in chapters 9 to 11. Field evaluation was done to determine whether clones developed from callus and/or cell lines with improved *in vitro* resistance have improved field resistance, but at the same time have agronomic characteristics similar to the original cultivar 'Late Harvest' cultivar. This would be to the advantage of the potato industry and similar methods could be developed for other diseases and resistance breeding programmes.

MATERIALS AND METHODS

Thirty-five clones of the potato cultivar 'Late Harvest' were selected from all the selected clones developed from cell and/or callus vines with *in vitro* resistance against *Fusarium oxysporum* culture filtrates and or fusaric acid as described in chapter 9, 10 and 11. The selection of the 35 clones was based on tuber shape, absence of malformation and leaf growth viability.

The 35 lines and a control (Late Harvest) were multiplied in the field during 1993 and 1994 and were planted in a randomized block design with three replications in October 1995. Thirty tubers were planted in 9 m rows. The interrow spacing was 1,0 m and intrarow spacing, 30cm. All the tubers were of the same physiological age and the same source. Fertilizer applications were based on regular soil analysis, while weeds, diseases and insect pests were controlled by chemicals registered for use on potatoes. Irrigation was scheduled according to a class A evaporation pan.

After harvesting all the lines were evaluated for their agronomic characteristics. They were evaluated according to size (small, medium and large), malformation of the tubers, secondary growth, eye depth, sandsplit, tuber shape and stolon indentation. Total marketable and unmarketable yields were also determined. Evaluations were done according to the following index scale values:

Malformation of tuber:

1 = absolutely malformed
2 = more than 10% malformed
3 = less than 10% malformed
4 = no malformation

Tuber size:

<100g = small
100-250g = medium
>250g = large

Eye depth

1 = deep

2 = medium

3 = shallow

Secondary growth

1 = 60 - 100% secondary growth

2 = 30-60% secondary growth

3 = 10-30 % secondary growth

4 = less than 10% secondary growth

5 = no secondary growth

Tuber shape:

1 = long

2 = rectangular

3 = oval

4 = round

Sandsplit:

1 = 60 - 100% sandsplit

2 = 30 - 60% sandsplit

3 = 10-30% sandsplit

4 = less than 10% sandsplit

5 = no sandsplit

Stolon indentation:

1 = reasonable indentation

2 = little indentation

3 = no indentation

After the agronomic characteristics were evaluated 20 unblemished, medium (100-250g) tubers were selected from each of the three replications and kept in brown paper bags at 4 to 5°C for 12 weeks. The laboratory assessment of potato tuber resistance to dry rot was conducted according to the method described by Theron & Holtz (1987). After the tubers were kept at room temperature for two days, inoculations were done by injecting a 0,2 cm^3 spore suspension (1 x 10^4 propagules cm^{-3}) into each tuber (Theron & Holtz, 1987). Tubers were placed in paper bags and kept at 25°C and 50-70% RH for 21 days to promote dry rot development. After incubation the tubers were cut in half and the extent of dry rot development determined according to a dry rot disease index scale (Theron & Holtz, 1987).

Analysis of variance for marketable and unmarketable yields were done according to Genstat 5 release 3.2 (1995). The mean values were tested for differences against the control cultivar, Late Harvest (7)* by means of Dunnett's multiple comparative test. SAS (SAS Institute INC. 1989) were used. Index values were given according to a specific agronomic characteristic of the potato, (these index values were summarised for each cultivar), the higher the value, the stronger the characteristic. These values were used to determine whether the clones differed agronomically form the control 'Late Harvest' by using the chi-square test. Genstat 5 release 3.2 (1995) was used for this analysis. Only clones which differed significantly from the control cultivar, 'Late Harvest' (7) were indicated, since only clones which performed better than the control would be acceptable for using in the potato breeding programme.

RESULTS AND DISCUSSION

Index values for different cultivars are shown in Table 12.1. Clone 166 had a significantly higher marketable yield than the control cultivar Late Harvest and the other clones ($P<0.005$) (Table 12.1). Except for clone 166 there were no statistical differences between the clones and the control cultivar for all the agronomical characteristics which indicated that the genotypes of the clones were not altered. These results are important since only clones which performs the same or better than the control cultivar would be included in the potato breeding programme. Although it was established that for the agronomical characteristics there were no statistical differences, it was important to determine whether the clones had improved resistance against *Fusarium*

oxysporum. Results obtained in field trials indicated that from the 35 clones, six performed significantly beter than the control cultivar 'Late Harvest' (Table 12.1).

The clones with the highest average degree of resistance against *Fusarium oxysporum* were clone 45 (index = 1,46), clones 86, 141 and 209 had resistance indexes of 1,39 and clones 166 and 228 index values of 1,37 (Table 12.1). The disease index for the "Late Harvest" control was 1,28; Steyn, Mckenzie & Visser (1991) reported an index value of 1,23 and Theron & Holtz (1987) 1,37 for "Late Harvest". There were no significant differences between the other clones and the control "Late Harvest". This evaluation must however be repeated in the Fusarium resistance breeding programme to determine the stability of the resistantce, before the clones could be included in the potato breeding programme. Other authors were also able to obtain disease resistant plants by using tissue culture. Toyoda *et al.,* (1984) selected tomato calli resistant to fusaric acid and Wenzel et al., (1984) used fusaric acid as a selective agent on barley. By the selection of cell lines resistant to toxins produced by the pathogen *in vitro* followed by regeneration of plants from resistant cell lines it was possible to obtain potato plants with improved resistance to *Fusarium oxysporum.* The same method also proved successful in producing tobacco plants resistant to *Pseudomonas tabacci* (Wolf & Foster) Stevens (Carlson, 1973), potato plants resistant to *Fusarium oxysporum* Schlecht. f. sp. *tuberosi* (Weimer) Snyder & Hansen (Behnke, 1980a) and *Phytophtora infestans* (Mont.) De Bary (Behnke, 1979; 1980b), maize plants resistant to *Helminthosporium maydis* (Nishikado & Miyake) race T (Gengenbach & Green, 1975; Gengenbach, Green & Donovan 1977) Brettell, Goddard & Ingram, 1980), *Brassica napus* L. plants resistant to *Phoma lingam* (Tode ex. Fr.) Desm. (Sacristan, 1982) and *Medicago sativa* L. resistant to *F. oxysporum* f. sp. *medicaginis* (Hartman, McCoy & Knous, 1984a,b).

Although six of the 35 selected clones had improved field resistance (14,2%), the success rate was relatively low, taken into consideration that only 35 from all the lines or clones with *in vitro* resistance could be evaluated, due to the fact that all the other clones were disqualified based on irregular tuber shape, malformation or poor leaf growth viability, prior to this evaluation. The cost and time implication of both conventional breeding methods and *in vitro* selection methods should be taken into account when determining whether the success rate is satisfying. The low

success rate may be explained by the fact that not all cell lines stably resistant to *Fusarium oxysporium*. filtrate, regenerated plants with increased resistance to *Fusarium in vivo.* The same phenotype, i.e. resistance to toxic filtrates, may be due to different genetic or possibly epigenetic changes in different lines. Daubt (1986) found that in selection for resistance against a crude pathogen filtrate one must isolate as many resistant cell lines as possible, since many of them may have been selected for resistance to components other than the putative toxin, but if enough are generated, some may carry the desired resistance. The variation may be explained by the strong selection pressure, which results in different types of mutation, acting at different stages of the reaction to a particular toxin or pathogen. This implies furthermore that genetic rather than epigenetic changes at the cellular level have taken place. It has to be checked by segregation experiments and the reaction of lines insensitive to the toxin has to be compared with their reaction to the pathogen (Chawla & Wenzel, 1987).

A question frequently discussed is the effect of the toxic filtrates used for selection on changes in ploidy of the regenerated plants. Hartman, *et al.*, (1984a,b) found polyploid plants with a greater *in vivo* resistance among regenerants from calli resistant to filtrate. The fact that the effect of gene dose may play a role in the increased resistance to pathogens is also mentioned by Latudedada & Lucas (1983). In the experiments with alfalfa a low level of resistance to *Verticillium albo-atrum* in parents was simply amplified in polyploid somaclones. Arcioni, Pezzotti & Damiani (1987) found increased ploidy among alfalfa regenerants after selection for *F. oxysporum* resistance. Apart from the length of action of the toxic filtrates the initial genotype used for selection also seems to play a role. As is shown by Alicchio, Antonioli & Palenzona (1984) in *Solanum melongena* (eggplant), toxins of pathogens may affect chromosome number in cultivated cells by interacting with cell genotypes. Binarová, *et al.*, (1990) used large scale selection in which they managed to obtain some alfalfa plants with increased resistance to *Fusarium* species. Hartman *et al.*, (1984a,b) and Arcioni *et al.*, (1987) selected callus cultures of alfalfa on a smaller scale, but achieved a higher number of resistant regenerated plants. This may be due to the different genetic backgrounds of the initial plant genotypes used for selection. Other authors indicated that the desired traits cannot be derived from any source of parental material and that in the case of disease resistance it would be better to start with the highest possible level of resistance in a desirable agronomic background (Daubt, 1986). In such material,

resistance can be increased much more effectively by *in vitro* selection than by simple screening of somaclonal variants of regenerated plants. These results and results obtained in this study support the concept of using cell culture technology to increase resistance to *Fusarium* species, but they revealed the need to learn more about the biochemical and physiological bases of the interaction of pathogen *versus* plant *in vivo* and in cell culture *in vitro*.

CONCLUSION

The value and advantage of the developed *in vitro* technique is that a large number of breeding lines can be screened in a relatively short period of time and in limited space, right through the year, which is not possible in conventional methods. Screened lines with better resistance should become part of the potato breeding programme. This method can help the breeder by reviving failed breeding lines or by allowing unexpected variation to be expressed which could allow novel-breeding material. Although *in vitro* inductions of mutations cannot replace cross-breeding, it can be valuable in supporting breeding programmes. It is apparent that *in vitro* approaches hold considerable potential for increasing the genetic diversity available to the breeder and hence would be of use in disease resistance breeding.

Table 12.1 Field evaluation of 35 potato clones with improved *in* vitro resistance against fusaric acid and/or culture filtrates of *Fusarium oxysporum* (<0,005) Statistical differences which were indicated are those which after statistically from the control cultivar, 'Late Harvest' (Clone 7). Differences between clones were not indicated, since only clones which performed better than the control would be acceptible for using in the breeding programme.

Clone	Mean Yield (Kg/ha)					Disease Index
	Small	Medium	Large	Total marketable	Unmarketable	
7	1.960	9.13	4.95	16.04*	4.04	1.28
166	5.133*	17.82*	5.09	28.04	3.21	1.37*
221	3.520	15.27*	5.93	24.73	2.49	1.27
191	2.870	12.94	7.79	23.60	1.54	1.29
47	3.443	12.31	6.66	22.41	2.67	1.28
162	3.333	12.29	5.86	21.48	1.21*	1.28
227	2.707	12.13	4.89	19.73	2.02	1.27
18	4.173	11.96	4.44	20.57	1.83	1.26
46	2.723	11.87	5.37	19.97	1.86	1.29
86	2.693	11.72	7.60	22.01	2.46	1.39*
135	4.153	11.62	7.15	22.93	1.59	1.28
231	2.613	11.45	6.75	20.89	1.84	1.28
30	2.393	11.09	6.30	19.78	1.57	1.26
228	2.650	10.56	5.72	18.92	1.50	1.37*
152	2.887	10.43	5.41	18.73	1.95	1.27
45	3.707	10.76	3.87	17.73	1.32*	1.46*
209	2.480	9.48	5.60	17.56	1.97	1.39*
61	2.767	9.26	6.32	18.35	1.82	1.31
222	1.613	9.00	6.83	17.45	1.65	1.30
95	1.843	8.66	5.08	15.59	1.37*	1.28
224	1.443	8.42	7.45	17.31	1.19*	1.26
234	1.923	8.38	8.58	18.88	1.85	1.28
235	1.390	8.31	3.86	13.56	2.92	1.31
31	1.877	8.14	5.13	15.15	1.43*	1.31
237	1.250	7.40	4.91	13.56	2.39	1.28
141	1.660	7.11	6.27	15.04	1.79	1.39*
185	2.230	6.95	4.06	13.24	1.95	1.27
74	1.257	6.88	8.98	17.12	1.40*	1.31
71	1.123	6.34	6.69	14.15	1.15*	1.26
164	1.363	5.54	7.36	14.26	2.00	1.27
91	1.167	5.23	1.89	8.29	0.87*	1.31
193	1.467	5.22	4.93	11.62	1.02*	1.31
230	0.794	5.11	4.98	10.89	0.65*	1.30
155	0.670	5.08	8.86	14.61	1.09*	1.29
110	1.521	3.91	1.35	6.79	2.03*	1.28

* The * indicate clones which differ significantly from the control cultivar, Late Harvest (7) at the 0,05 level.

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*DEVELOPMENT OF AN *IN VITRO* SELECTION TECHNIQUE OF POTATO CULTIVARS FOR IMPROVED RESISTANCE TO *FUSARIUM OXYSPORUM*

by

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RESUME

Fusarium oxysporum W.C. Snyder & Hans. causes dry rot, stem-end rot and wilt of potatoes (*Solanum tuberosum L.*) Screening for Fusarium resistance is important and an effective screening method is essential. The aim of the study was therefore to develop an *in vitro* selection and/or screening technique to select and/or screen potato somaclones with improved resistance to *Fusarium oxysporum.* Techniques for the *in vitro* production, cultivation and regeneration of five South African potato cultivars, Late Harvest, BP1, Vanderplank, Up-to-Date and Kimberley Choice, have been established and optimized. It was important to develop an optimal *in vitro* system which could enable one to add selective agents (toxin and/or culture filtrates) to screen the potato cultivars for better resistance against *F. oxysporum.* A correlation was found between fusaric acid production and virulence of isolates of *F. oxysporum.* The toxin, fusaric acid, seemed to play a major role in the development of dry rot in potato tubers of susceptible cultivars. Virulence, Vegetative compatibility's and RAPD analysis have classified *F. oxysporum* isolates that cause dry rot on potatoes into groups that coincide with races. All three techniques proved useful in differentiating among isolates of *F. oxysporum* and due to cost effectively RAPD analysis may be the more widely applicable technique especially for screening large samples of isolates. The toxicity of *F. oxysporum* culture filtrate and fusaric acid on cell and callus cultures of the

different resistant and susceptible cultivars have been described. It was found that an *in vitro* relationship existed between susceptibility of potatoes to *F. oxysporum,* and toxicity of fusaric acid and culture filtrates of *F. oxysporum.* Results suggested that fusaric acid could be used as selective agent. It is possible that approaches described in the study will have an impact on the production of novel disease - resistant potato plants particularly when conventional breeding methods have failed. The extent to which it can be exploited will, however, depend on whether the recovered undesirable heritable trials could impede the breeding process. Plants regenerated from callus cultures resistant to *F. oxysporum* culture filtrate and/or fusaric acid have been selected and evaluated for agronomic potential as well as field resistance against *F. oxysporum.* The main advantage of the developed *in vitro* screening techniques remains the large number of individuals which can be screened in a small space in a relatively short period of time, right through the year and the potential source of novel breeding material for trails. Results obtained in this study warns that the applying of *in vitro* selection for the improved resistance of potato cultivars to *F. oxysporum* by adding only one characteristic without altering the remaining genotype should be researched carefully. It can be anticipated that *in vitro* selection and screening for disease resistance as a breeding tool will continue to expand in future. This method is however dependent on linkage to the convential breeding program. This linkage will ensure that the screened and developed tissue culture material has the agronomic performance that is essential.

ONTWIKKELING VAN 'N *IN VITRO* SELEKSIETEGNIEK VIR AARTAPPEL-CULTIVARS VIR VERBETERDE WEERSTAND TEEN *FUSARIUM OXYSPORUM*

deur

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SAMEVATTING

Fusarium oxysporum W.C. Snyder & Hans. veroorsaak droëvrot, stingel-end vrot en verwelk van aartappels (*Solanum tuberosum* L.). Evaluering vir Fusariumweerstand is belangrik en 'n effektiewe evalueringsmetode is noodsaaklik. Die doel van die studie was daarom om 'n *in vitro* seleksie en/of evalueringstegniek te ontwikkel om aartappelklone en -cultivars met verbeterde weerstand teen *Fusarium oxysporum* te evalueer en/of selekteer. Tegnieke vir die *in vitro* produksie, kultivering en regenerering van vyf Suid-Afrikaanse aartappel cultivars, Late Harvest, BP1, Vanderplank, Up-to-Date en Kimberley choice is ontwikkel en geoptimaliseer. Dit was belangrik om 'n optimale *in vitro* sisteem te ontwikkel, sodat selektiewe agente (toksiene en/of kultuurfiltrate bygevoeg kan word om aartappelcultivars vir verbeterde weerstand teen *F. oxysporum* te evalueer. Daar was 'n korrelasie tussen fusaarsuurproduksie en virulensie van isolate van *F. oxysporum.* Dit blyk dat die toksien, fusaarsuur, 'n belangrike rol in die ontwikkelking van droëvrot van aartappelknolle van vatbare cultivars kan speel. Virulensie, vegetatiewe verenigbare groepe en RAPD analise het die *F. oxysporum* isolate wat droëvrot veroorsaak in groepe verdeel wat ooreenstem met rasse. Al drie hierdie tegnieke is handig vir die differensiering van isolate van *F. oxysporum* en as gevolg van koste-effektiwiteit kan die RAPD analise moontlik meer toepaslik wees, veral vir die evlauering van 'n groot aantal isolate. Die toksisiteit van *F. oxysporum* kultuurfiltrate en fusaarsuur op sel- en kalluskulture van die

verskillende weerstandige en vatbare cultivars is beskryf. Daar is gevind dat daar 'n *in vitro* verwantskap tussen vatbaarheid van aartappels vir *F. oxysporum* en toksisiteit van fusaarsuur en kultuurfiltrate van *F. oxysporum* is. Resultate wys daarop dat fusaarsuur as selektiewe agent gebruik kan word. Dit is moontlik dat die benadering wat in die studie beskryf word 'n impak op die produksie van nuwe siekteweerstandbiedende aartappel plante kan hê, veral waar die konvensionele telingsmetode gefaal het. Die mate waartoe dit gebruik kan word sal egter daarvan afhang tot hoe 'n mate die ongewenste erfbare eienskappe wat verkry is, die telingsproses beïnvloed. Plante wat van kalluskulture wat weerstandbiedend teen kultuurfiltrate van *F. oxysporum* en/of fusaarsuur is, geregenereer is, is geselekteer en geevalueer vir agronomiese potensiaal en veld weerstand teen *F. oxysporum*. Die hoofvoordeel van die ontwikkelde *in vitro* evaluerings tegnieke is die groot aantal individue wat in 'n klein spasie, in 'n relatiewe kort periode van tyd, reg deur die jaar evalueer kan word. Nog 'n voordeel is die potensiele bron van nuwe telingsmateriaal vir eienskappe wat moontlik nie deur konvensionele telings metodes verkry kan word nie. Resultate wat in hierdie studie verkry is, waarsku egter dat die toepassing van *in vitro* seleksie vir verbeterde weerstand van aartappelcultivars teen *F. oxysporum*, deur die byvoeging van slegs een eienskap, sonder om die oorblywende genotipe te verander, versigtig nagevors moet word. Daar kan verwag word dat *in vitro* seleksie en evaluering vir siekteweerstand as 'n telings hulpmiddel in die toekoms sal uitbrei. Hierdie metode is egter afhanklik van verbinding met die konvensionele teelprogram. Hierdie verbinding sal verseker dat die weefselkultuurmateriaal wat geevalueer en/of geselekteer is oor die agronomiese eienskappe beskik wat noodsaaklik is.

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