Studies on cocoyam (*Xanthosoma* spp.) in Nicaragua, with emphasis on *Dasheen mosaic virus*

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Abstract

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Cocoyam, *Xanthosoma* spp., is cultivated in tropical regions as a source of carbohydrates for human nutrition, for animal feed, and cash income for the farmers. It is the most important export crop of all the root and tuber crops grown in Nicaragua, but the national yield and total planted area have drastically decreased during the last few years. The crop has received little research attention, and previous to the studies presented in this thesis there was hardly any information available regarding the number of genotypes cultivated in the country, the agronomic performance of the different genotypes, which diseases that infect the crop, and to what extent these diseases influence the yield.

Three cocoyam genotypes were evaluated regarding agronomic traits in four field locations during two years. Significant interactions between genotype and location as well as between genotype and year were found for many of the traits. A differential response of the genotypes to the varying climatic conditions at the locations is one of the possible causes of these interactions. Different harvest times for the three genotypes are suggested, due to differences in time to reach the maximum growth. *Dasheen mosaic virus* (DsMV) is the most important virus that infects cocoyam. Virus-free plants were produced through meristem culture, and protocols for the regeneration of plants via callus and protocorm-like structures were established. Although a genotypic response to tissue culture was observed, all the tested genotypes produced shoots from meristem and from meristem-derived calli.

Under field conditions the virus-free plants performed better, regarding yield, than infected *in vitro* plants. The fact that the plants were virus-free could not, by itself, explain the yield increase, suggesting that the process of *in vitro* re-invigoration also played an important role. *Aphis gossypii* was the only winged aphid observed during the field trial, suggesting that this insect is the virus-transmitting vector.

cDNA fragments containing the coat protein (CP) and 3'-UTR regions of nine DsMV isolates were molecularly characterised. One isolate had a 19 aa long deletion in the N-terminus of the CP region. The Nicaraguan isolates formed two subgroups correlated with geographic origin of the cocoyam genotypes.

Key words: Cocoyam, genotype × environment interaction, DsMV, virus-free plants production, effect on yield, molecular characterisation.

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A la memoria de mi adorado padre Guillermo, el hombre más lindo del mundo, y a la memoria de mí siempre querido y recordado hermanito Jorge Luís.

Contents

Appendix

The present thesis is based on the following papers, which are referred in the text by their Roman numerals.

I. Reyes-Castro, G., Nyman, M. & Rönnberg-Wästljung, A.C. 2005. Agronomic performance of three cocoyam (*Xanthosoma violaceum* Schott) genotypes grown in Nicaragua. *Euphytica*, *142*, 265-272.

II. Reyes, G. & Nyman, M. Direct and indirect plant regeneration from meristem and shoot apex of cocoyam (*Xanthosoma* spp.) *Manuscript*.

III. Reyes, G., Rönnberg-Wästljung, A.C. & Nyman, M. 2006. Comparison of field performance between *Dasheen mosaic virus*-free and virus-infected *in vitro* plants of cocoyam (*Xanthosoma* spp.) in Nicaragua. *Experimental Agriculture, 42(3)*. *In press*.

IV. Reyes, G., Ramsell, J., Nyman, M. & Kvarnheden, A. Molecular characterisation of *Dasheen mosaic virus* isolates from cocoyam (*Xanthosom*a spp) in Nicaragua. *Manuscript*.

Introduction

Cocoyam (*Xanthosoma* spp.) is one of the six most important root and tuber crops world-wide (Jennings, 1987; Onwueme & Charles, 1994). The corm, cormels, and leaves of cocoyam are an important source of carbohydrates for human nutrition, animal feed (Ndoumou *et al.*, 1995; Nyochembeng & Garton, 1998) and of cash income for farmers (Tambong, 1997). The crop is mainly cultivated by small-scale farmers (Onwueme & Charles, 1994) in Asia, Africa and Latin America (Wilson, 1984). In spite of its importance as a staple food in many countries, cocoyam has received very little research attention (Goenaga & Heperly, 1990), and is regarded as an under-exploited, and insufficiently studied crop (Nguyen & Nguyen, 1987; Giacometti & León, 1994; Watanabe, 2002). In the beginning of the 1980's Nicaragua started to export cocoyam, which motivated the expansion of the production areas and the introduction of new genotypes into the country. The cocoyam production rapidly became an important support for the domestic economy of small-scale farmers, but in the last few years the production area has drastically declined (INTA, 2000).
According to Goenaga & Chardón (1995) the yield potential of cocoyam is seldom realized, mainly because of a lack of knowledge concerning diseases, proper management practices, and physiological determinants that may limit plant growth and development. In this respect, the studies presented in this thesis will hopefully contribute to a sustainable cocoyam production in Nicaragua.

Botanical characteristics and ecology

Cocoyam is an herbaceous, monocotyledonous crop. The main stem is a starch-rich underground structure called corm from which offshoots, termed cormels, develop. The sagittate-ovate leaves are between 1-2 m long and arise directly from the corm, with long ribbed petioles (Figure 1). The leaves have a marginal vein and two large basal lobes with variable pigmentation (Purseglove, 1972). Flowering is rare, but when it occurs, the inflorescence consists of a cylindrical spadix of flowers enclosed in a 12-15 cm spathe. The flowers are unisexual with the female flowers located at the base of a spadix and the male flowers at the top. Between the pistillated and the staminated flowers, sterile flowers are located. Flowering is more prone to occur in wet regions (Purseglove, 1972). The inflorescence of *Xanthosoma* is protogynous, and the pistillate flowers are normally receptive 2 to 4 days before pollen is shed (Wilson, 1984). The spadices are seldom fertile and produce few viable seeds (Giacometti & León, 1994).
Cocoyam is a perennial crop, but for practical purposes it is harvested after 9-12 months of growth. The growth and development cycle can be divided into three main periods. During the first two months the growth is slow. This period starts with the sprouting of shoots and ends when the cormels emerge. The second period is characterised by a rapid increase in shoot growth, until 6-7 months after planting (MAP), and it is during this period that the plants achieve their maximum leaf area, pseudostem diameter and height. During the third period the leaves start to wilt and the total dry weight of the plant above ground decreases until harvest.

This is the moment of major movement of photo-assimilates from leaves to the corm and cormels (Wilson, 1984; López, Vásquez & López, 1995). The senescence of the plant, at the end of this period (*ca*: 9-10 MAP) is used by farmers as a harvest index.

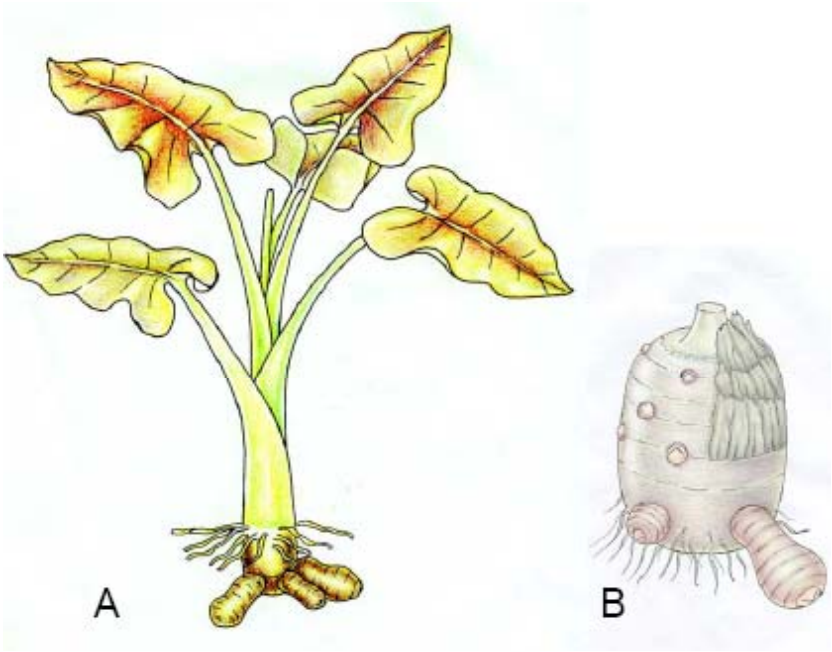

Figure 1. A) Cocoyam plant, B) corm and cormels.
(Drawing by Maycol Acuña Pérez).

Cocoyam is a strictly warm weather crop (Onwueme & Charles, 1994) that has become pan-tropic (Wilson, 1984). It is cultivated in tropical and subtropical zones, between latitudes 30 degrees north and 15 degrees south of the equator (López, Vásquez & López, 1995). It is not adapted to waterlogged conditions, but even though it is a lowland crop (Onwueme, 1978) it grows well in upland/rain-fed regions with well-drained soils and in a climate where rainfall (1400-2000 mm) is well distributed throughout most of the year. It grows more vigorously in adverse conditions, tolerating a certain amount of shade and heavier or drier soils than taro, another tropical aroid (Bown, 2000; Mathews, 2002). The crop can be grown in a range of pH of 5.5 to 6.5 and temperatures between 20 to 35ºC. Temperatures lower than 18 ºC slow the leaf growth; temperatures higher than 35ºC increase the foliage, but limit the corm and cormel formation. The general growth is favoured when the night temperature is between 14 to 29 ºC. Under these conditions the production of carbohydrates is increased (López, Vásquez & López, 1995).

Origin and taxonomy

Cocoyam is the only aroid species native to the American continent that is widely used for food (Bown, 2000), and it was domesticated and cultivated already during pre-Columbian times (Wilson, 1984; Montaldo, 1991). According to Bown (2000), cocoyam reached West Africa between the 16th and 17th centuries and was spread further by traders, missionaries and other travellers. It soon became more popular in African cultivation and diet than the "old" cocoyam (taro, *Colocasia esculenta*) that had been introduced from Southeast Asia. During the 19th century cocoyam was introduced into Asia and the Pacific, and into North America (Bown, 2000).

The etymology of the genus *Xanthosoma* comes from the Greek, xanthos = yellow, and *soma* = body, due to the yellow or yellowish colour of the corm and cormel pulp characteristic of several species (Mayo, Bogner & Boyce, 1997). *Xanthosoma*, with 57 species distributed in tropical and southern subtropical America, belongs to the tribe *Caladieae*, of the family *Araceae* (Mayo, Bogner & Boyce, 1997; Bown, 2000). This family contains two other genera cultivated for their edible corms and cormels, *Alocasia* and *Colocasia*.

The taxonomic position of the cultivated *Xanthosoma* species is unclear, and in recent years the tendency has been to give the name of *X. sagittifolium* to all cultivated *Xanthosoma* (Giacometti & Léon, 1994). However, some taxonomists have used leaf shape, pigmentation, and other vegetative characteristics to distinguish several additional species, including *X. violaceum* Schott (synonym *X. nigrum)*, *X. atrovirens* Koch & Bouché and *X. caracu* Koch & Bouché (Wilson, 1984).

Onokpise *et al*. (1999) describe three types of *X. sagittifolium* cultivated in Cameroon ("white", "red or pink" and "yellow"), based on the texture and colour of the corms and cormels and the colour of the petioles. "Red" and "white" cocoyams were able to hybridise, suggesting that they are varieties of the same species. According to Bown (2000) there are two main species, *X. sagittifolium* and *X. violaceum*. This division into species is based on the colour of the corm, cormels and leaves and on the shape of the cormels. The foliage of *X. violaceum* is purple-flushed and the corms and cormels are purple-grey with reddish eyes and purple, red, pink, yellow or white flesh. *X. sagittifolium* has green leaves and the corms and cormels have white, yellow or pink flesh and pale brown skin. The shape of the cormels from *X sagittifolium* is globose, and for *X violaceum* ovate-elliptic (Bown, 2000). The cocoyam grown in Nigeria was for many years presumed to be *X. sagittifolium*, but is now classified as *X. mafaffa* (Bown, 2000).

The common names for cocoyam are numerous and diverse, including tannia, tannier, yautia, malanga, new cocoyam (Wilson, 1984), ocumo, mafafa, rascadera, tiquisque, quequexque, calusa, mangarito, tayobe, taye, macabo (López, Vásquez & López, 1995), quiscamote, mangarás and taioba (Giacometti & León, 1994). In Nicaragua cocoyam is known as quequisque, and close to the border of Honduras, quiscamote.

Cultivation practices

Before planting, the soil needs to be ploughed and furrowed to facilitate the growth of the cormels. Mounds can also be formed for planting. The portion of the corm or cormels are placed with the buds downward in the furrows and covered with a 5-7 cm layer of soil.

The time of planting depends on the water availability. At planting the soil needs to be moist, and if an irrigation system is available, the planting can be made the whole year around (Wilson, 1984). However in traditional farming systems, the planting is done immediately when the rains become regular. The crop requires moisture throughout its growing season (6-7 months). If water is supplied after this period, plant growth resumes and the time for harvest can be extended. For some genotypes an extension of the growing seasons reduces the commercial yields as a result of cormel sprouting (Goenaga & Chardón, 1993). On the other hand, insufficient water applied during the growing period retards the development of cormels and causes yield reductions (Caesar, 1980).

The conventional planting material includes portions of the central corm (100-150 g), cormels, or whole small cormels. Corms and cormels display a dormancy-like period of approximately five weeks, during which sprouting does not occur (Wilson, 1984). The removal of the apical dominance, by destroying the apical bud, accelerates the sprouting of the lateral buds.

Cultural practices, such as the distance between plants and between rows, that result in increased leaf area or leaf area index, usually also result in increased yield (Wilson, 1984). It is a common idea that close spacing between plants increase the corm and cormel yield per hectare, but the yield per plant is higher at wider spacing (Wilson, 1984; Onwueme & Charles, 1994). This relationship, can however be modified depending on the time when the crop is harvested. Wider spacing results in reduced yield per hectare when harvesting is made before 9 MAP, but increases the yields when harvesting is delayed until 12 MAP (Wilson, 1984). The relation between plant density and time of harvest may be related to the water and nutrients available in the field. In Nicaragua, 0.6 × 0.8 m between plants and furrows (≈ 16,000 plants ha^{-1}), or 0.6 × 1.0 m (≈ 17,000 plants ha^{-1}) are the most common spacings used.

Fertilisation. Cocoyam responds well to organic and chemical fertilisation (Karikari, 1971; Giacometti & León, 1994). In fertile soils the crop develops lush leaves and produce higher yields. The leaf growth and cormel yield are increased by the addition of fertiliser, and high fertilisation reduces the time required for cormels to reach the maximum size (Chandler, Irizarry & Silva, 1982; Wilson, 1984). Split fertilisation at planting, and 2, 4 and 6 MAP is used when the harvest takes place 9-12 MAP (Chandler, Irizarry & Silva, 1982; Giacometti & León, 1994), and one additional fertilisation at 12 MAP is required if cocoyam is grown as a biennial crop (Chandler, Irizarry & Silva, 1982).

Weed control. Weed control is a beneficial cultural practice during the early stage of the growing period (Purseglove, 1972). Especially when the cormels start to grow, weed competition has a detrimental effect on yield (Sangakkara, 1992). Hand or chemical weeding is used during the period before the canopy is formed.

Depending on the conditions, machetes or hoes are used. Plants require to be earthed up several times; weeds are then controlled, the exposure of roots is prevented and the development of the cormels favoured.

Harvest. Harvest usually occurs during the dry season (Onwueme & Charles, 1994) but cormels can be harvested anytime during the year. The time from planting to harvest varies with genotype and method of cultivation. In commercial plantations harvesting usually takes place between 9-12 MAP (Onwueme & Charles, 1994; Bown, 2000), but cocoyam harvested at 14-15 MAP has also been reported (Purseglove, 1972; Goenaga & Chardón, 1993). In very humid locations in Cameroon cocoyam is grown as a biennial or perennial crop, and multiple harvesting is practised. Under these conditions yield vary from 12 to 37 tonnes ha^{-1} depending on the place, conditions and method of production (Onwueme & Charles, 1994).

The leaf area or leaf area index has consistently been shown to correlate with corm and cormel yields (Wilson, 1984; Agueguia, 1993). Any drastic reduction of the leaf area, mainly during the second period of the growth and development cycle, results in reduced corm and cormels production.

Cocoyam is generally grown by small-scale farmers (Onwueme, 1988), but plantations under intensive management can also be found. Since cocoyam tolerates shade, the crop is frequently grown in intercropping systems together with permanent crops such as banana, coffee, coconut, rubber, oil palm and cocoa (Wilson, 1984; Bown, 2000).

Diseases

Dasheen mosaic virus

Dasheen mosaic virus (DsMV) is the most important viral pathogen of cultivated aroids world-wide (Chen *et al.,* 2001), and was first reported by Zettler *et al.,* in 1970. In Central America DsMV was first identified in Costa Rica by Ramírez (1985). DsMV is classified as a species of the recognized genus *Potyvirus*, family *Potyviridae* and consists of a flexuous filamentous particle (>700 nm) containing a positive sense single-stranded RNA genome (Figure 2).

The visible symptoms on the plants includes leaf distortion, vein chlorosis, mosaic feathering along the veins (Zettler, Jackson & Frison, 1989), and in case of a severe attack, stunted plants (Figure 3). DsMV is transmitted to a non-infected plant in a non-persistent manner exclusively by aphids (Brunt *et al.,* 1996) and the virus transmission in the field can be very rapid, probably in less than one hour (Pernezny, Lambert & Ramos, 1993). Although DsMV is not lethal, it retards the plant growth and reduces the yield (Zettler, Jackson & Frison, 1989).

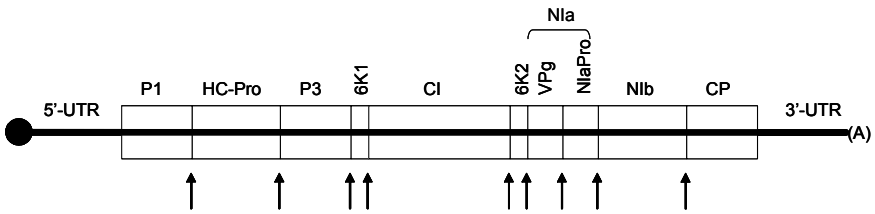

Figure 2. Genome organisation of the family *Potyviridae,* except for the genus *Bymovirus* (Adams *et al.,*2005). The single open reading frame is flanked by a untranslated region (UTR) at both ends, and encodes 10 mature proteins: P1 protein (P1), helper component proteinase (HC-Pro), third protein (P3), 6 kDa protein (6K1), cylindrical inclusion protein (CI), 6 kDa protein 2 (6K2), nuclear inclusion protein a (NIa), which is further processed into the viral genome-linked protein (VPg) and the main viral proteinase (NIaPro), nuclear inclusion protein b (NIb) and the capsid protein (CP). The functions of the proteins are diverse, but all coding regions and the 5' and 3' UTR are indispensable for virus propagation.

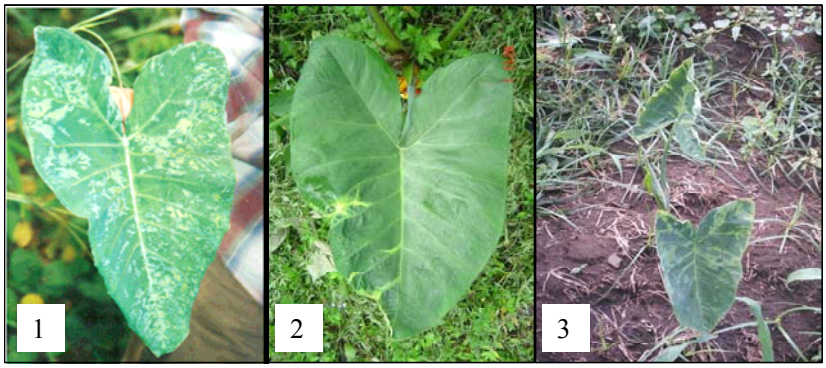

Figure 3. Symptoms of DsMV in cocoyam. 1) "Feathering" along the veins, 2) leaf distortion, 3) stunted plant.

Root rot disease

The root rot disease (RRD) is the most devastating disease in cocoyam at present (Tambong, Sapra & Garton, 1998) and can cause a total loss of yield (Saborío *et al.,* 2004a). The symptoms are stunting, yellowing of the foliage, and a reduction or elimination of the root system. Several different pathogens have been reported to be associated with the RRD including *Rhizoctonia* (Giacometti & León, 1994),

Sclerotium rolfsii (Bejarano-Mendoza *et al.,* 1998) and *Fusarium* spp (Saborío *et al.*, 2004a). However, the pathogen that seems to be the main causal agent is *Pythium myriotylum* (Nzietchueng, 1984; Pacumbaba *et al*., 1992; Onwueme & Charles, 1994; Tambong, Poppe & Höfte, 1999).
The disease is spread through soil and planting material (Nzietchueng, 1984) and can persist in the soil for many years. Chemical control, wide spacing, high mounds, regulation of the time of planting (Onwueme & Charles, 1994), drainage improvement, use of disease-free planting material (Saborío *et al.,* 2004b), planting on ridges, crop rotation (Giacometti & León, 1994), and use of organic fertiliser (Torres-Portuguez, 1996) have been suggested in order to control the disease, but with unsatisfactory results.

Conventional breeding and tissue culture

Cocoyam is a neglected crop mainly grown by small-scale farmers, and attempts to improvement have therefore been limited. The crop rarely flowers and the flowers are protogynous, which makes the use of classical breeding methods difficult. Successful induction of flowers, after treatment with gibberellic acid, has been reported (Alamu & McDavid, 1978; Alamu*,* McDavid & Duncan, 1982; Tambong & Meboka, 1994) and a few attempts to combine different genotypes through sexual crossings have been made (Goenaga & Hepperly, 1990; Onokpise *et al.,* 1999). However, no new improved varieties have, so far, been reported from these experiments.
Tissue culture techniques open up many possibilities for sustainable production and improvement of crops. Meristem culture of cocoyam has been reported (Tsala, Omokolo & Balange, 1996; Zok *et al.* 1998) and meristem-derived plants perform better in terms of yield than virus-infected *in vitro* plants (Reyes, Rönnberg-Wästljung & Nyman*,* 2006). According to Zok, *et al.* (1998) cormels from meristem-derived plants grow faster and the tuberisation starts earlier in comparison with conventionally propagated plants. An additional advantage of the *in vitro* plants is the re-invigoration frequently experienced in plants passed through a tissue culture phase (Pierik, 1990). Meristem-derived plants are also important for a safe exchange of germplasm between countries.
Due to the fact that cocoyam is vegetatively propagated, the Nicaraguan germplasm is preserved under field conditions. This approach is risky, since diseases or natural catastrophes can cause the loss of genetic resources. *In vitro* storage of cocoyam under minimal growth conditions (Zandvoort, Hulshof & Staritsky, 1994) and cryopreservation of *in vitro* shoot tips of taro (Takagi *et al.,* 1997) has been reported. These techniques are suitable alternatives to the field collections.
Tissue culture also gives the possibility to generate new genotypes through somaclonal variation, and a variety of morphological changes in callus-derived cocoyam plants has been reported by Gupta (1985). The genetic variation can be increased by chemical or physical mutagenic agents. Plants with different levels of tolerance towards the RRD, has been obtained through the irradiation with gamma rays of *in vitro* grown apices (Saborío *et al.,* 2004b).

Tetraploid induction, using colchicines, has been reported (Esnard *et al*., 1993; Tambong, Sapra & Garton, 1998), but no evaluation of the resulting plants has been reported. Since classical breeding has failed to produce new improved genotypes, alternative methods such as genetic transformation and protoplast fusion, is worthwhile to explore. So far, only transient expression of the reporter gene ß-glucuronidase (GUS) after particle bombardment of embryogenic calli has been reported (Hidalgo *et al.,* 2000)

The importance of cocoyam world-wide

In developing countries root and tuber crops (R&T) are very important, especially in the food systems of remote, generally marginalized areas, with particular low income levels (Scott, Rosengrant & Ringler, 2000a). Under traditional farming systems, R&T crops have some advantages over other sources of carbohydrates. They produce the highest yields of calories per unit of land area (Jennings, 1987; Agueguia, 2000), the yields are stable under conditions where other crops may not succeed (Scott, Rosengrant & Ringler, 2000b), and they are cheap to produce (Jennings, 1987). Besides being a source of carbohydrates, R&T crops contain proteins and vitamins and serve as a food security crop, alleviating seasonal shortages of food caused by natural or man-caused disasters (Scott, Rosengrant & Ringler, 2000a). According to Scott, Rosengrant & Ringler, (2000b) R&T crops will play multi-purpose roles in the global food system as a starch supplier, food security crop, source of cash income, as raw material for feed and processed products, and as key components in small-scale agro-enterprise development.

Cocoyam is one of the major R&T crops of the world. It is ranked sixth in planted area and production (Onwueme & Charles, 1994) after cassava (*Manihot esculenta*), potato (*Solanum tuberosum*), sweet potato (*Ipomoea batatas*), yam (*Dioscorea* spp.) and taro (*Colocasia* spp.). The main areas of cultivation of cocoyam include the Caribbean region (West Indies, Puerto Rico, Cuba and Dominica), Central and South America, Hawaii, West Africa (Nigeria, Ghana, Cameroon and Togo), tropical Asia (Malaysia, Indonesia, and the South Pacific Islands) and Florida (Purseglove, 1972; Tambong *et al.,* 1997; Bown, 2000). Cocoyam contains 15-26% of carbohydrates (Onwueme & Charles, 1994), and has a nutritional value comparable to potato (Giacometti & León, 1994). The crop has a relatively high protein content compared to, for example, cassava, yam, potato and sweet potato. Depending on genotype, the protein content has been estimated to be between 2.5% to 9.4% (Onokpise *et al*., 1999). This is important since cocoyam is a staple crop and as such is consumed in relatively large quantities. The vitamin C content of cocoyam is also favourable compare to many other root and tuber crops. Onwueme (1978) reported a vitamin C content of 96 mg/100g, while taro contains approximately 10 times less. In addition cocoyam contains ß-carotene, niacin, riboflavin and significant amounts of thiamine (Onwueme, 1978; Tambong, Poppe & Höfte, 1999).

The cocoyam production is almost exclusively used for human consumption (Onwueme & Charles, 1994), and all parts of the plant can be eaten. Due to minuscule bundles of crystals of calcium oxalate, that have an irritating effect, corms and cormels are prepared by boiling, roasting, or baking (Agueguia, 2000).

Flours are occasionally prepared, and in some areas the leaves are consumed as a green vegetable (Wilson, 1984; Jennings, 1987; Bown, 2000).

Cocoyam in Nicaragua

Cocoyam is a one of the most ancient crops cultivated in Nicaragua. It was already consumed extensively during the 17th century by misquitos and sumos (Wheelock, 1998), ethnic groups settled in the Caribbean coast. Cocoyam is known as "duswa" by miskitos and "wilis" by sumos. Until the late nineteen seventies, cocoyam production was devoted to local consumption, and three main zones of traditional cultivation could be identified: the central pacific (Masaya), the central-north (Nueva Segovia), and the Caribbean (Bluefields, Puerto Cabezas) (Figure 4). In Nicaragua, cocoyam is traditionally consumed boiled in soup, mixed with other vegetables and either with pork, chicken, fish or beef. Peeled and boiled cormels are also eaten by pounding the pulp with butter, milk, spices and salt. In the Caribbean coast of the country, a pie based on cocoyam, and a traditional fish dish "roundown" are prepared (Wheelock, 1998).

Figure 4. Map of Nicaragua. The Pacific area has volcanic soil, the most fertile in the country. The central and north regions are highland areas. The rain forest is located in the Caribbean part of the country.
(Drawing by Maycol Acuña Pérez)

In the beginning of the eighties, an increased international demand for cocoyam motivated farmers in non-traditional production areas to start to cultivate the crop. Farmers in the south Caribbean rain forest area (Nueva Guinea), the north Pacific dry areas (Chinandega) and the central highland zones established small commercial areas to expand the crop production for export (Figure 4). The export of cocoyam from Nicaragua has since then steadily increased, with a minor

reduction in 2002 (Table 1). The crop is exported mainly to Puerto Rico, the United States and Costa Rica (MIFIC, 2005), but also to Panama, Liberia and Belgium (MAGFOR, 2000).

Table 1. Volume and values of the cocoyam export in Nicaragua.

Year	Volume*	Value**
1994	1 692	775
1995	1 993	1 032
1996	2 341	865
1997	2 543	2 238
1998	2 534	2 543
1999	4 170	2 251
2002	3 875	1 765
2003	4 700	2 119
2004	5 509	2 747

* Thousands of kg ** Thousands US$

Cocoyam is to date an important crop for Nicaraguan agriculture, especially for the economy of small and medium-scale farmers located in the humid zones. In the year 2004 it was the third most consumed root and tuber crop after potato and cassava, and the second regarding production and use of arable land after cassava (MAGFOR, 2005). During the same period cocoyam was the most important export product of all root and tuber crops grown country-wide (CEI, 2005). In spite of its importance for the country, the total area has steadily decreased from 30,000 ha in 2001 (MAGFOR, 2003) to 6,450 ha in 2004 (CEI, 2005). Furthermore, the national average yield has declined from 19-22 t ha^{-1} in 1999 (INTA, 2000) to 7.2 t ha^{-1} in 2004 (MAGFOR, 2005)

Aims of the study

- Evaluate the agronomic performance and DsMV incidence of cocoyam grown in Nicaragua.
- Produce DsMV free plants from meristem culture, and produce and regenerate plants from adventitious shoots and callus culture.
- Evaluate the effect of DsMV on yield and the rate of re-infection.
- Molecularly determine the relationship between DsMV isolates from cocoyam collected in two production areas in Nicaragua.

Results and discussion

Collection and characterization of Nicaraguan *Xanthosoma* germplasm (unpublished results)

Cultivated and wild *Xanthosoma* were collected from different areas in Nicaragua. In total six cultivated, one ornamental and nine wild plants were found (Figure 7). The cultivated genotypes were distinguishable both at the morphological and at the molecular level. In a study using PCR-based RAPD, they all showed different banding patterns. The chromosome number of all cultivated genotypes was 2n = 26 (Figure 5).

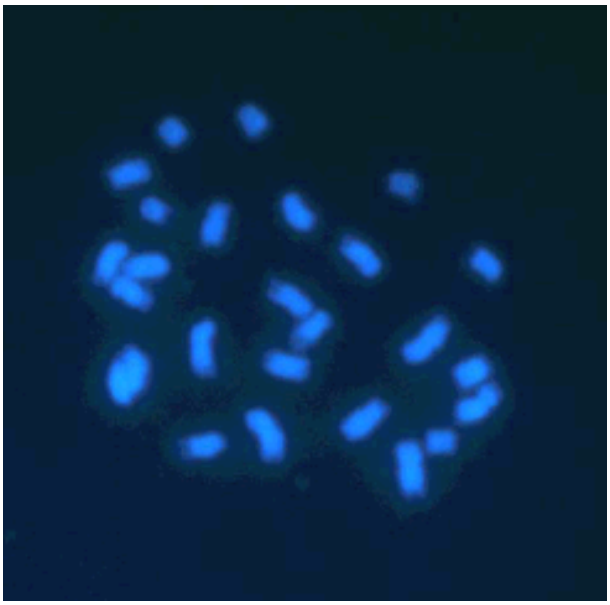

Figure 5. DAPI-stained chromosomes from cultivated cocoyam.

The genotype Nueva Guinea (NG) was introduced into Nicaragua in the mid-80´s from Costa Rica and was named by farmers according to the name of the area where it was introduced. The origin of the genotype Masaya (MY) is unknown, but it has been cultivated in the Masaya area for at least 100 years. In the northern part of Nicaragua (Nueva Segovia) a genotype, which the farmers refer to simply as quequisque (the Nicaraguan name for cocoyam), was found. This genotype was introduced into the country from Panama at the beginning of the 90´s and we named it Apalí (AP) after a village nearby. One additional genotype was found cultivated in Chinandega (west Pacific) that we named according to the volcano Casitas, situated in the area. The origin of this genotype is unknown. All the above-mentioned genotypes have purple-flushed petioles and green leaves. The cormels are ovate-elliptic and the flesh of the cormels ranges from pink to reddish-

purple (Figures 6 and 7). All the cultivated genotypes with white-fleshed cormels are called Blanco. The leaves of Blanco are light green and the petioles are slightly purple-flushed at the base. The shape of the cormels is asymmetric, with a globose "head" and a "tail" (Figure 6 and 7). Blanco shows many of the features described for *X. sagittifolium*, while the remaining genotypes fit more into the description of *X. violaceum*. There are clearly some discrepancies and uncertainties regarding the taxonomy at the species level within the genus *Xanthosoma*, and a revision of the genus is evidently needed.

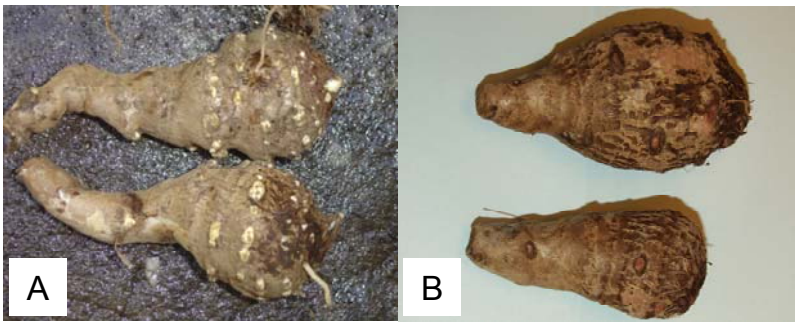

Figure 6. Shape of the cormels in cultivated cocoyams. A) Blanco, B) Apalí.

The old traditional genotype, MY, seems to have some advantages over the recently introduced genotypes. MY can be harvested at the time when Nicaraguan farmers usually harvest cocoyam. The consumers prefer this genotype because, in contrast to other genotypes, the cormels keep their pink-purple colour after boiling. Furthermore, MY has a porous texture after cooking, while the other genotypes have a more doughy consistency. Sellers prefer MY because its cormels can be stored for a longer period without deteriorating compared to cormels from other genotypes. This is also an important quality when the crop is exported. In general, MY produces a slightly lower number of cormels, but they are, on the other hand, heavier than cormels produced from other genotypes. The ornamental plant was classified as *X. atrovirens monstrosum*. Nicaragua is situated in the center of origin of *Xanthosoma* and nine wild cocoyam relatives were found. Three of the plants were classified as *X. wendlandii* and one as *X. mexicanum.* These two species have previously been reported to be found in the country (Croat & Stiebel, 2001). The remaining plants have not yet been classified.

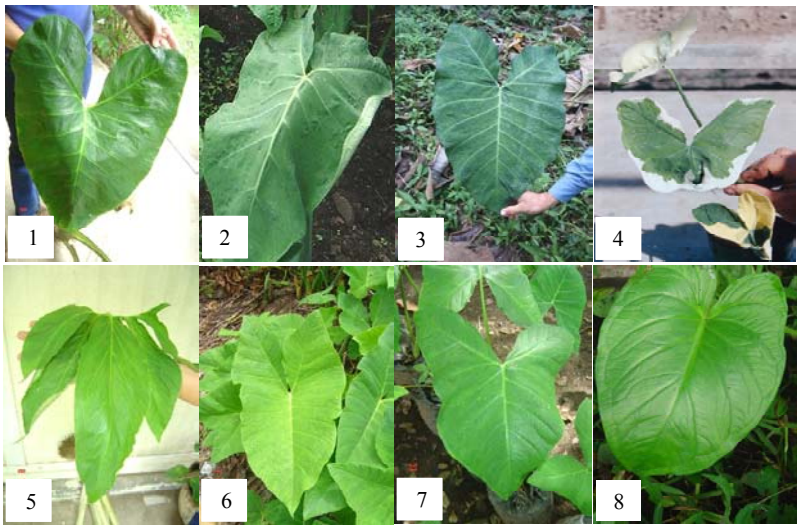

Figure 7. Cultivated genotypes; 1) Casitas, 2) Blanco, 3) Nueva Guinea, 4) *X. atrovirens monstrosum*. Wild species; 5) *X. wendlandii*, 6) *X* spp., 7) *X.* spp., 8). *X. mexicanum.*

Field performance of three cocoyam genotypes under cultivation (Paper I)

Morphological variables and yield. The analysis of differences in field performance between the genotypes MY, NG and AP grown in the locations Masaya, Nueva Guinea, Managua during 1999 and 2000 and in Nueva Segovia in 1999 are summarised in Table 2. Statistically significant differences between genotypes for the different traits were mainly found in Masaya, in both years, and in Nueva Segovia.

Table 2. Evaluated effect and statistical differences found after analysis of variance of phenotypic (5 traits) and yield component data (5 traits).

Evaluated effect	Statistical differences (number of traits)
Genotype at each location and year	Masaya: 1999 (7) and 2000 (6) Managua: 1999 (0) and 2000 (0) Nueva Segovia: 1999 (6) Nueva Guinea: 1999 (1) and 2000 (3)
G × L during each year	1999: (7) (all yield components) 2000: (5)
G × Y in each location	Masaya: (5) Nueva Guinea: (2) Managua: (1)
L × Y for each genotype	MY: (7) NG: (5)

The specific climatic conditions at the different locations are possible causes for the ranking shift and the different responses of the genotypes from one year to the other (G x Y interaction) and between the locations (G x L interaction) during each year. The rain forest conditions in Nueva Guinea, where the rainy season lasts 9-10 months, the highland condition in Nueva Segovia, the drought period in the early growing season in Masaya in 1999, and the RRD incidence in Nueva Guinea in 2000, can be a part of the explanation for the different interactions found in this study.

The yield was higher in Nueva Guinea, where the 1400-2000 mm year^{-1} rainfall required for optimal growth and development of cocoyam (Onwueme & Charles, 1994; Goenaga, 1994) is easily achieved. The unpredictable and variable climate in Masaya is one of the main factors influencing yield across years at that location. In Managua, a non-traditional cocoyam area, the rainfall is insufficient for cocoyam production.

Physiological maturity. Differences between the genotypes in the time to reach the maximum leaf area and plant height, and in the number of cormels with sprout and roots at harvest (9 MAP) (Figure 8), indicate that the genotypes need to be harvested at different times after planting. On average NG and AP reached the maximum leaf area three weeks earlier than MY. At harvest NG and AP showed a higher percentage of cormels with sprouting roots and buds than MY in year 2000.

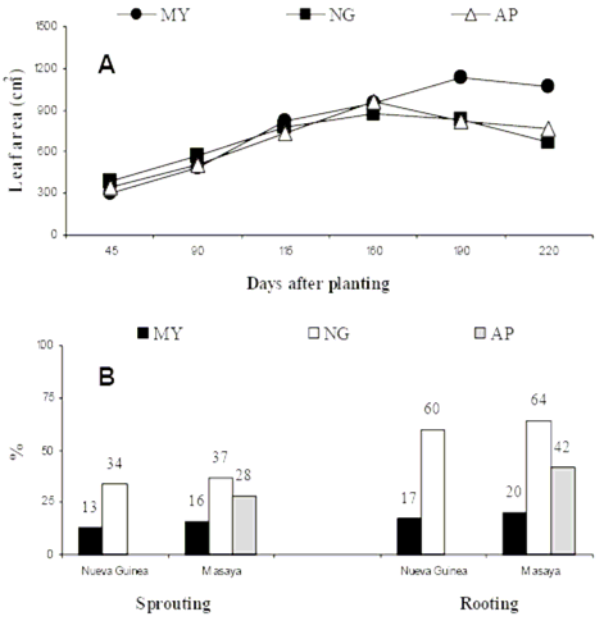

Figure 8. A) Development of the leaf area of the largest leaf in Masaya, 1999. B) Percentage of cormels with roots and buds sprouting at harvest in Nueva Guinea and Masaya 2000.

Yield losses and deterioration of the quality of the cormels are related to starch degradation that occurs during the rooting and sprouting of the cormels. To obtain optimal yields it is very important to determine the time at which each genotype should be harvested. Nine MAP seems to be a rather suitable time for the old traditional genotype MY, but is unsuitable for NG and AP.

DsMV incidence. The ELISA test indicated that plants showing the feather-like symptoms associated with DsMV in fact contained the virus. In general, the actual number of plants with virus infection was much higher than the screening for visible symptoms indicated. The intermittence of the expression of the symptoms masks the actual number of infected plants; plants without symptoms could still be infected by the virus (Figure 9).

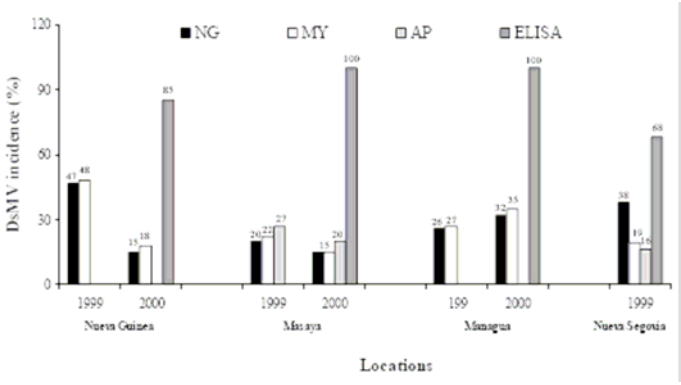

Figure 9. Percentage of plants with visible symptoms of DsMV and infection percentage according to ELISA.

The percentage of plants infected with dasheen mosaic virus differed across locations but not between genotypes. The higher percentage of infections found in Managua and Masaya compared with Nueva Guinea and Nueva Segovia might therefore be due to differences in the climatic conditions between the test sites, which in turn affected the aphid population density and the DsMV transmission. High temperatures and low rainfall are ideal conditions for aphid reproduction and movement (CIP, 2002), conditions that are fulfilled in Masaya and Managua.

Tissue culture for virus-free plant production and potential plant breeding (Paper II)

This study was addressed to produce DsMV-free plants, to study the tissue-culture response of different genotypes (NG, MY and Bco), and to create a baseline for further studies in plant improvement.

Meristem culture. After 90 days in culture the percentage of meristems that produced shoots varied according to genotype. Depending on hormone concentration in the media, 92-100% of the MY explants produced shoots, while the meristems isolated from NG and Bco were more dependent on the

concentration of the external addition of plant growth regulators for shoot production. The highest percentage of regenerating explants from NG (83%) was achieved when 1 mg l^{-1} of IAA in combination with 0.5 mg l^{-1} of BAP was added to the medium. The regeneration capacity of meristems from Bco varied from 8-100%, with the highest percentage of shoot production recorded on hormone-free medium. The ELISA test showed that 94% of the plants of MY and 100% of the plants regenerated from NG and Bco meristems were DsMV-free.

Plant regeneration from protocorm-like bodies. Spherical structures known as protocorm-like bodies (PLBs) developed on all media evaluated, except on the hormone-free medium. Media containing 6-BAP induced a larger number of PLBs than media containing kinetin. At high concentration of kinetin (10 mg l^{-1}) 60% of the explants from MY, 43% from NG and 36% from Bco, became necrotic. After transfer to hormone-free medium, between 33-100% (depending on genotype and initiation medium), of the PLBs developed into plantlets. In general, PLBs that had been produced on high concentration of hormones were the most reluctant to develop into plantlets, except for Bco grown on 10 mg l^{-1} 6-BAP, where 92% of the PLBs produced developed into plantlets.

Plant regeneration from calli. Calli were obtained from meristems and shoot apices but not from leaves or petioles. Calli were produced from meristems on all culture media evaluated. In cultures of meristems from the genotypes MY and NG the highest percentage of explants producing calli (48 and 69%, respectively) was recorded on medium containing 3 mg l^{-1} 2,4-D. Higher concentrations of 2,4-D or 2,4-D in combination with kinetin, drastically reduced the number of callus-producing explants. In meristem cultures of the genotype Bco the concentration of hormones had no pronounced effect.

In the shoot apex cultures the lower concentration of 2,4-D (3 mg l^{-1}), with or without kinetin added, yielded calli, while media containing 5 mg l^{-1} 2,4-D only produced a few calli in one genotype. Sixty days after transfer of the calli to hormone-free media, 94%, 83% and 58% of the calli from MY, NG and Bco, respectively, produced shoots.

Effect of DsMV on yield (Paper III)

A field trial was established in Managua aiming to evaluate the agronomic performance and the effect of DsMV on yield of virus-free (NI) and virus-infected (I) *in vitro* plants. With this purpose repeated measurements of morphological variables and ELISA tests to evaluate the virus re-infection, were made during the experimental period.

Morphological variables and yield. Independently of infection status or genotype the height of plant, leaf area and diameter of the pseudo-stem increased continuously up to 217 days after planting (DAP) (around 7 months), after which the growth curves declined. With a few exceptions where NI plants were higher (124 and 151 DAP) and had a larger leaf area (124 DAP) and higher number of leaves (189 DAP), no statistically significant effect was found for genotype or infection status regarding growth of the plants, although NI plants showed higher numerical values for all traits during the evaluation time. The vegetative growth of these *in vitro* propagated plants agrees well with the vegetative growth of

conventionally propagated plants, characterised by a rapid increase in shoot growth until 6-7 MAP, after which the leaves start to wilt and the total dry weight decreases until harvest.

No significant difference in yield was found between genotypes, although MY and Bco showed the heaviest cormels, Bco and NG produced the largest number of cormels, Bco developed the longest cormels, and MY produced the widest cormels. The yield per plant in NI plants was significantly higher than yield obtained in I plants. The estimated yield ha^{-1} for NI plants was 18.2 t and 13.4 t for I plants. The yield per plant and the estimated yield ha^{-1} obtained in this study were several times higher than in the previous study (study I), mainly due to an increase in the number of cormels per plant. This enhanced cormel production might be associated with the process of *in vitro* re-invigoration (Pierik, 1990), a phenomenon often experienced when plant material has been passed through a tissue culture phase.

The NI plants produced 25% higher yield compared to the I plants. Since NI and I plants were handled in a similar way, the lower yield obtained from I plants is most probably due to the effect of the accumulation of viruses. The I plants were obtained from field plants that had been exposed to infection and re-infection through many propagation cycles and it is likely that the NI plants have to be re-infected with the virus during several cycles to show a reduction in yield.

The estimated yield obtained from NI plants (18.2 t ha^{-1}) was 2.5 times higher than the current national yield average (7.2 t ha^{-1}), and very close to the former national yield average (19-22 t ha^{-1}) reported by INTA in the year 2000. Since the plants in this study were produced in a non-traditional area it is very likely that production would increase if the plants were grown in a traditional area with enough water supplied.

Virus re-infection and vector monitoring. The numbers of NI plants that were re-infected with DsMV increased up to 192 DAP (≈ 6 MAP) when the percentage of re-infected plants with DsMV was highest in Bco (90%) compared to MY (65%) and NG (60%). Insects from 18 families and 6 orders were identified in the field trial. *Aphis gossypii* was the only winged aphid collected in the study. Several aphid species have been shown to be effective vectors of DsMV, including *Aphis gossypii*, *Myzus persicae* and *Aphis craccivora* (Simone & Zettler 1991). Since *Aphis gossypii* was the only aphid recorded in this study it was most probably the transmitter of DsMV during the field experiment.

Molecular characterization of DsMV isolates (Paper IV)

Samples, with visible symptoms of DsMV, were collected from three cocoyam genotypes cultivated in two production areas, Masaya (MY) and Nueva Guinea (NG).

Specific primers were used to amplify the coat protein (CP) and 3´-untranslated (3´-UTR) regions of nine DsMV isolates (NiNG1, NiNG3, NiNG18, NiNG67, NiMY70, NiMY72, NiMY75, NiMY76 and NiMY78). The fragments were cloned into a pGEM®-T Easy Vector (Promega, Madison, WI, USA) and sequenced.

Sequencing of the cloned RT-PCR product for isolate DsMV-NiNG1 revealed that it contained almost the complete CP region (nt 1-918) and 3'-UTR (nt 919-1211). DsMV-NiNG1 showed the highest nt identity (96%) to DsMV-LA from *Colocasia* in Florida (Pappu *et al*., 1994b). A relatively high sequence identity was also found to other DsMV isolates, such as DsMV-M13 (Chen *et al*., 2001) at 86%, and to the *Vanilla mosaic virus* isolate VaMV-FP at 76%. Phylogenetic analyses of the nucleotide and deduced amino acid sequences of DsMV-NiNG1 and 7 isolates of the BCMV group within the genus *Potyvirus* (Adams *et al*., 2005) showed that DsMV-NiNG1, DsMV-M13 and VaMV-FP formed a well-supported clade (bootstrap value 100%). DsMV-NiNG1 and DsMV-M13 grouped together in the DsMV/VaMV clade.

Partial CP sequences (534 nt) were determined for eight other Nicaraguan DsMV isolates from cocoyam. Sequence comparisons among the Nicaraguan isolates showed that the nt and aa sequence identities varied from 80 to 100% and from 85 to 100%, respectively. The isolates from the region Masaya, all shared high sequence identities (98-100% at nt and aa level). Isolates NiNG1, NiNG3 and NiNG67, from the region Nueva Guinea, showed 97-99% nt and aa identities to each other. NiNG18 was most divergent of the NiNG isolates and displayed 87-88% nt identity to the other NiNG isolates and 80% nt identity to NiMY isolates.

Comparison of the Nicaraguan isolates with previously published DsMV isolates showed that NiNG3 and NiNG67 shared the highest nt identity (97-98%) with DsMV-LA (Pappu *et al*., 1994b). Also for NiNG18, the nt identity was highest to DsMV-LA (87%). The NiMY isolates shared 86-87% nt identity with DsMV-LA and DsMV-DK, isolated from Japanese *Colocasia* (Chen *et al*., 2001).

In the N-terminus of the CP region, all Nicaraguan isolates, except NiNG18, shared a 10 aa sequence repeated three times in tandem, previously reported to be present in DsMV-LA (Pappu *et al*., 1994b). NiNG18 showed a 19 aa long deletion, which included the first 10 aa repeat. Differences in length of the CP coding region is a common characteristic in potyviruses (Shukla *et al*., 1994), and this has been reported also for DsMV (Chen *et al*., 2001; Li *et al*., 1998; Shi *et al*., 2005).

Phylogenetic analyses using partial nt sequences for the CP coding region of Nicaraguan DsMV isolates, previously reported DsMV isolates, *Zucchini mosaic virus* (ZYMV) and VaMV isolates, showed that the DsMV and VaMV isolates formed a monophyletic group (bootstrap value 99%). The Nicaraguan isolates were placed within the DsMV clade, confirming that they belong to this species. The Nicaraguan DsMV isolates formed two subgroups: one subgroup with the NiMY isolates and another subgroup with the NiNG isolates. The NiMY subgroup was well supported (bootstrap value 100%) and these isolates showed a very close relationship. Except for NiNG18, there was also a strong support in the analysis for the NiNG subgroup (bootstrap value 99%). The phylogenetic analysis placed DsMV-LA in the NiNG subgroup.

The different origins of the cocoyam genotypes grown in the country might explain the genetic diversity within the Nicaraguan isolates. The NiNG isolates were collected from the genotypes Nueva Guinea and Blanco, both recently introduced from Costa Rica. The NiMY isolates were collected from the genotype Masaya that has been cultivated in the area with the same name for more than 100 years.

The fact that DsMV-LA was isolated at a location geographically close to Nicaragua, (Florida, USA), could explain the close genetic relationship between the NiNG isolates and DsMV-LA.

General discussion and future perspectives

In 1975, the National Academy of Sciences defined cocoyam as a neglected food crop with economic potential, and it is still regarded as an under-exploited, and insufficiently studied crop (Nguyen & Nguyen, 1987; Giacometti & León, 1994; Watanabe 2002). In Nicaragua cocoyam has been cultivated since pre-colonial times, and has in recent years become an important export crop for the country, especially for farmers in humid and rain forest areas.

Previous to these studies hardly any information was available regarding the number of genotypes cultivated in the country, the agronomic performance of the different genotypes, which diseases infected the crop, and to what extent these diseases influence the yield. The recent introduction of planting material from other countries has been conducted without previous knowledge of the genetic background or diseases that are spread through the corms and cormels.

The Nicaraguan cocoyam production has relied on the use and re-use of vegetatively propagated material without any process of sanitation or re-invigoration. This recurrent infection of the planting material has lead to a gradual decrease in yield over the years. DsMV has been shown to be present in all commercial plantations in Nicaragua (Reyes, Nyman & Rönnberg-Wästljung, 2005), and the yield loss caused by the virus has been estimated to be around 25% (Reyes, Rönnberg-Wästljung & Nyman, 2006). In terms of money for the small-scale farmers this yield reduction is substantial.

The lack of resistant genotypes makes the production of clean planting material the only immediate possible solution to increase the yield and maintain the production areas. However, the low price of cocoyam in the local market makes production based directly on *in vitro* plants non-profitable. A more economically viable alternative would therefore be to distribute planting material in the form of cormels produced from *in vitro* plants to the farmers. These *in vitro* plants should then be grown in areas with no or little inoculum pressure (Figure 10).

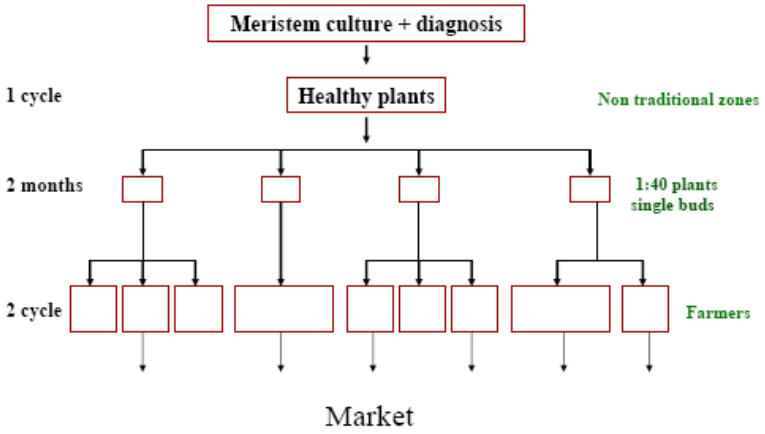

Figure 10. Scheme for the use of disease-free plants obtained from meristem culture.

In order to optimise the cormel production from *in vitro* plants, and thereby reduce the cost per plant, Valverde *et al.* (1997) suggested a delay of harvest time from normally 9-10 MAP to 12-14 MAP. The cormels produced will be used for propagation and not for consumption; and the sprouts and roots that develop from the cormels when harvest is delayed (Reyes, Nyman & Rönnberg-Wästljung, 2005) is therefore not a problem. Each cormel usually has five to ten buds and the corm between 15-30 buds that have the potential to develop into new plants. The use of pieces of corms/cormels with a single bud for propagation would maximise the number of plants from each mother plant. Each generation in field involves the risk of disease re-infection, so high multiplication rate at initial stage is important. The multiplication using single buds will allow 40 new plants to be obtained from a mother plant.

An interesting, and more permanent approach would be to produce DsMV-resistant plants. In many plant species, virus-resistant plants have been obtained through transformation with the coat protein coding region of the virus (Powell *et al*., 1986; Germundsson, 2005). In this study the coat protein coding region from DsMV was isolated, and this information could be of further use in the production of transgenic plants resistant to the virus. Since it is quite easy to regenerate plants from meristems, the production of transgenic plants through particle bombardment of the meristems or meristem-derived calli would be feasible.

There are strong evidences that the RRD is present in the rain forest region close to the Costa Rican border, and this disease is most probably the main reason why the total area cultivated with cocoyam has decreased during the last few years. When the harvest started to decline farmers moved to new areas in an attempt to increase the yield, but since they were using already infected planting material the disease was spread. The use of disease-free planting material is therefore of utmost importance as a way to prevent the spreading of the disease to new areas in the region and to other regions in the country. The steady increase in cocoyam export (Table 1) shows the importance of the crop to the farmers' economy. Therefore a national program, aimed at producing and distributing disease-free planting

material, is required for the sustainable improvement of cocoyam production in Nicaragua.

Regarding the possible RRD in the rain forest area, the pathogens involved need to be identified, the plant-pathogen interaction studied, and ways to control the disease evaluated.

The cocoyam production in Nicaragua is relying on few genotypes, and research with the aim of evaluating disease tolerance/resistance of different genotypes, including wild cocoyam relatives, would be valuable. The difficulties encountered with classical breeding makes alternative methods, such as genetic transformation, tetraploid induction and somatic hybridization attractive alternatives.

The information generated in the studies presented here can be used to optimise the agronomic management of the crop. Distributing disease-free planting material and informing the farmers about the diseases that attack cocoyam could prevent further spreading of the possible RRD. These studies also form a base for further studies in plant breeding, including genetic transformation.

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