Newsletter

ISSUE NO. 8 **JULY, 2003**

IN THIS ISSUE

From the Editor's Desk:

In this issue, an eclectic range of subjects is covered by authors familiar to the INGENIC Newsletter readership. The underlying message is that cocoa researchers are working in exciting times. At a time when *sustainability in cocoa production* are buzz words, research results are reflecting that cocoa research programme planners are cognisant of the need for productive, superior planting materials with resistance to the myriad of diseases and pests, and thus suited to environmentally friendly management practices.

Moreover, the CFC Project on *Cocoa germplasm conservation and utilisation: a global approach* has demonstrated that much progress can be made through consensual planning of cocoa research activities and collaboration. All of the participants in this Project, many of whom are members of INGENIC, deserve commendation.

The upcoming INGENIC and INCOPED Workshops and the International Cocoa Research Conference should provide fora for a continuation of this mutually beneficial cooperation. The INGENIC Committee looks forward to successful outcomes of these meetings, and the guidance they will provide for it to continue realising its mandate to foster links among cocoa breeders, geneticists and institutes, and promote progress in cocoa breeding, genetics, germplasm evaluation and collaborative research.

The INGENIC Committee wishes to express its appreciation to the contributors to this issue of the Newsletter. We encourage other cocoa scientists to use this medium to communicate with the rest of the cocoa fraternity. Please submit articles for publication to me at louisebekele@yahoo.co.uk, louisebekele@hotmail.com or fbekele@fans.uwi.tt on or before May 30th, 2004 in time for the release of the electronic version at the end of June and the publication of the hard copy in July.

In this issue, the term 'cacao' is used to denote the tree and its parts and 'cocoa', dried beans and the commercial product.

The INGENIC Committee wishes to thank ACRI and BCCCA for financial support of INGENIC activities. We also acknowledge BCCCA, CIRAD, CRIG, CRU, MCB & UESC for logistical support. The Editor is very grateful to Dr. Elizabeth Johnson and USDA for the kind donation of a computer and peripherals for the morphological characterisation project at CRU, and the preparation of INGENIC documents.

Thank you for your continued support and interest in this newsletter.

Best wishes!

Frances Bekele

Who Needs Clothing?

R. Lockwood

Yoel Efron and the Miami Cocoa Genetics Group debated approaches to the development of better cacao varieties (INGENIC Newsletter Issue 7, pages 36 and 37). They are in agreement that resources are limited and should be used in the most cost-effective way. Their argument is about what approaches are most likely to bring results. Making the right choice depends on understanding both the changing world of cocoa production and the true status of cocoa breeding, including the reasons for that status.

The background

The social and economic backgrounds of cacao cultivation are changing. An income of less than $1/ day for many cocoa farmers is unacceptable. Increase in this income is more likely to come from higher yields than from higher prices, given continuation of the long-term declines in the price of agricultural products. The crop has been described as dependant on a "forest rent", but preservation of that forest is becoming a consumer requirement. This is a cost to the farmers, and again can be met only through higher returns from cultivating the crop. Those same consumers are demanding increasingly tighter restrictions on the use of crop protection chemicals, and some are opposing the use of fertilisers. This puts further demands on the farmers.

On the fragile soils of West Africa, cocoa is a mature industry with all the signs of declining yields in the longer-established areas. Historically, as cocoa production has declined in one area, new frontiers have opened. This situation is changing too, with few new frontiers of cocoa development around the world and none with decisive natural advantage in cocoa production as currently understood. Variety and agronomy packages are needed that will allow continuing cocoa production in the traditional producing countries, at the same time as meeting the consumer's requirement of socially and environmentally friendly production practices.

In small and large-scale agriculture alike, higher profits are most often achieved by increasing yield per unit area because many production costs are related to the area of land that is cultivated. Intensifying production is consistent with the increasing pressure on the land for biological production of all kinds, in response to population pressure and the necessity to use renewable resources whenever possible. More productive varieties are a prerequisite to the socially and environmentally sustainable cocoa systems of the future. They are required for the transition from a way of life to a business, on which development depends.

Cocoa breeding achievement to date

What has been achieved through scientific approaches to cacao improvement over the last fifty years? Hunter (1990) reviewed the status of cacao in the Western Hemisphere. He observed "The tragedy of cacao growing in the Western Hemisphere today is that, outside of a few varieties, most of which have not been subjected to rigorous testing, little is currently available for farmers in the way of superior planting materials". Few would argue that the situation is markedly different in Africa, where the "hybrids" have not lived up to the promises made of them and appear not to have been widely adopted anyway. In the Far East and Oceania, plantation cocoa has largely disappeared because the financial returns were too low and the management problems too great to make the crop profitable in sustained periods of low prices. Cocoa breeders have not delivered what today's farmers require.

Domesticating cacao

Clones are the most efficient means of exploiting genetic variation, which is why they are the planting material of choice whenever they are technically and financially feasible. Among the tropical crops, rubber and then tea switched to clones, and oil palm is trying to follow suit. In cacao, clones are a key step towards the domestication of the crop: the change to plagiotropic growth habit combined with a suitable balance between crop and continuing growth of the trees brings the pods within sight and reach of the farmer, greatly simplifying pest and disease management. The side-grafting technique (Yow and Lim, 1994) and variations of it are a low cost if technically demanding way of upgrading genetically unproductive trees by using them as root-stocks for locally productive scions.

The better parents will be drawn from the pool of better clones, though good clones are not necessarily good parents. Creating better cacao varieties depends on advances in clone selection, whether for seedling or clonal planting material.

Measuring achievement

In January 2002, two experienced cocoa breeders were asked to identify their "150 best cacao clones" for use in further breeding. There was a fair measure of agreement between them with 189 clones identified. The origins of these clones, including the approximate decade in which a particular clone was identified or selected, are summarised in the Table below.

Origin	Decade in which Selected									
	20	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**Unknown**	**Total**
Cross	2	0	8	7	15	3	1	0	0	36
Cultivated	12	15	12	18	13	3	0	0	0	73
Uncultivated	0	50	2	4	9	6	0	0	0	71
Unknown	0	0	0	1	0	1	0	0	7	9
Total	14	65	22	30	37	13	1	0	7	189

The table shows that fewer than 20% of the "best" clones originated from any form of breeding (as opposed to selection) programmes. Thirty-seven percent of the clones are of uncultivated origin that is taken from "wild" cacao and a further 38% from early generation cultivated cacao. Only 7% of the clones originated in the last 30 years, and almost half of these were of uncultivated origin. Clone selection has received limited attention or proved unduly difficult in recent decades. This is the root cause of the failure of cocoa breeding

The contrast with oil palm and rubber is illuminating. Both crops developed from genetic bases, which most cocoa breeders would consider dangerously narrow. Hardon, Corley and Lee (1987) estimated that oil yield had increased from 2.8 to 4.5t/ha over four generations of selection among palms derived from the original four Deli *duras*. Tan (1987) showed that breeding from Wickham's seedling material had led to a four-fold yield increase by the mid-eighties. Perhaps half of the increases were due to agronomy and the other half to breeding.

The philosophy of main stream cacao improvement

For much of the last fifty years, most of the "scientific" cocoa breeding has been linked with predictive tests of disease resistance. Bartley (1986) concluded that "Breeding for disease resistance in cacao has been, on the whole, very unsuccessful". Reviewing breeding for disease resistance in Trinidad, Simmonds (1993) wrote "There is no doubt, from first principles, resistance to all these diseases could be built up over generations by methods familiar in a multitude of crops; and, wherever cacao is efficiently bred, no doubt it will be built up. The general failure noted by Bartley (1986) reflects a long-term failure of comprehension of the facts that a VR (vertical resistance) immunity against an airborne fungus is a mirage and that HR (horizontal resistance) is always constructible. It must surely be significant that the very competent breeding programmes that are now in place in South East Asia quickly identified HR to black pod and vascular streak dieback as the bases for control".

More recently, the philosophy has extended to exploration of an ever broader genetic base. Twelve years ago, Warren and Kennedy (1991) argued that "There is more than adequate genetic variability already available to breeders to produce vast improvements in yield". The writer is not aware of any economically important trait in cacao where an extended search of germplasm has led to identification of the much desired quantum leap in its expression. Diverse examples include resistance to swollen shoot virus, cocoa butter content and combining ability for yield.

Why not breed for yield?

All of the early selection programmes were directed towards higher yield, through the selection of clones. However, the high yield of crosses involving F.J. Pound's Upper Amazon material, explained as heterosis, led to much intercrossing from the fifties onwards. After a few years, Bartley (1967) observed that the yield of a clone was no guide to its value as a parent. If this was so, how can one select one's parents? There is no breeding methodology. Correcting defects, using predictive tests of disease resistance, became attractive. When progress proved to be slow, the response was to broaden the genetic base.

A re-interpretation of breeding for yield

From the mid-eighties onwards, abundant evidence accumulated that the inheritance of yield in cacao is strongly additive. If yield is inherited additively, why isn't the yield of a clone an indicator of its general combining ability for yield, as it is in all other crops? The answer lies in the variation in optimum planting density of the material commonly used by breeders. Even within the Upper Amazon material, this ranges from well under 1,000 trees/ha for vigorous clones such as PA 76 to perhaps as high as 5,000 trees/ha for Amazon15-15, both under near ideal climatic and soil conditions. As the discussion of root-stocks in INGENIC Newsletter 7 shows, the natural variation in the optimum planting density of cacao is not fully appreciated. Once clones are grown at their optimum planting density, their yields become as useful indicators of gca for yield as they do in any other crop.

And heterosis? The idea arose from observations among seedling material, some of Upper Amazon origin, transferred from Trinidad to Tafo, Ghana in 1944. There was no formal test of heterosis: it was simply an interpretation of observations. Certainly there is evidence of inbreeding depression. However, the preliminary results of critical tests of heterosis in crosses among Upper Amazon crosses reproduced as clones in Sabah were equivocal. The mean vigour mostly exceeded mid-parent values (MPVs), but there was no gain in yield efficiency. There was no pattern to the departures from MPVs, which were not consistent with current concepts of the genetic distance between the parents.

Empirical proof of breeding for yield

Three cocoa breeding programmes stand out around the world: Edwin Freeman's well known one with the Trinidad Cocoa Board, which led to the TSH series of clones, the programme modelled on rubber breeding started by Ron Shepherd with Harrisons and Crossfield (now Golden Hope Bhd) in Malaysia, which led to the PBC clones, and Humberto Castro's programme in Ecuador, which led to the CCN clones. All generated excellent clones in a short period, from simple crossing programmes and selection for yield in the field. There was no concern about scientific correctness – none of the programmes led to major scientific papers. John Anselmi, a plantation owner identified BR 25, the highest yielding clone in Sabah in a simple hierarchical selection programme in commercial cacao. He started with over 900 candidates.

These examples demonstrate that the methods applied in other crops work just as well in cacao. The reason for the widespread failure of cocoa breeding is simple: proven breeding methods have not been used enough.

So who needs clothing?

The future of cocoa depends on the establishment of breeding and agronomy programmes which generate the combinations of varieties and husbandry packages that will maintain the profitability of cacao cultivation through continuing social, economic and environmental change. Such programmes depend far more on practical skill in cacao cultivation, especially with clones, and the ability to manage very large numbers of field trials with small experimental errors, than they do on advanced scientific knowledge. The scale of an effective programme was given by Chong and Shepherd (1986).

The Common Fund for Commodities project entitled *Cocoa germplasm utilisation and conservation: a global approach* has made a useful contribution to the development of skills with clones in West Africa. The farmer selection programmes proposed for phase two are a further step in the required direction. However, reports from the CFC project show that skills with clones require further development. As Chong and Shepherd show, the scale of the programmes needs to be many times larger than they are today to ensure success.

The progress made by conventional breeders of most tropical tree crops has largely passed cacao by. Cacao needs to catch up, and it is quite clear how this can be done, however unglamorous the process. The process is not exclusive to practical breeders and agronomists – it will be much more efficient if backed by appropriate high quality science.

Encouraging cocoa research directed at bettering the farmers' lot requires a paradigm shift, away from fashionable science which might contribute at some stage, to competent practical breeding and agronomy which identifies for itself what science is required in support. It also requires a shift from reward through publication to reward for better varieties grown by farmers. The next step is to build those practical programmes, which are judged and rewarded by their results for farmers. In this way, the cocoa research community, as well as the donors, will be decently clothed.

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Survey on the Growing Practices and Planting Material used for Cacao Growing in the Central Region of Cameroon

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Abstract

In 2001, a survey was conducted among a limited number of cocoa producers in central Cameroon in order to gain a clearer picture of their cultural practices and of planting prospects in an unfavourable context after State withdrawal from the commodity chain and a period of low prices. This study provided a description of the planting material grown, and ascertained what producers thought of it. The survey confirmed the dominant role that cacao growing still plays for smallholders in the central region of Cameroon, who had no intention of converting their cacao plantings to any other crop, and who regularly carried out replacement planting in their plantations and were even considering extensions in the short term. Very little of the material grown came from varieties developed by research. There were two reasons for this: a level of resistance to pod rot that was judged insufficient, and the inefficiency of seed distribution systems. The generalised use of endogenous seeds slowed down the progress in pod rot control that could have been provided by genetic improvement. However, if producers could be convinced of the merits of improved varieties, whose precocity, yields, and particularly their level of resistance to pod rot had been proven, they would be prepared to pay for such varieties.

Introduction

There are two major cacao-growing zones in Cameroon, Centre-South, which contains the Centre and South provinces, along with the Sanaga Maritime department in the Littoral province, and Southwest, which contains the Southwest province and the centre of the Moungo department in the Littoral province. There is also small-scale cocoa production in the East, more particularly in the Boumba and Ngoko and Haut Nyong departments.

Annual production peaks at around 120,000 t for plantations estimated to cover 400,000 ha, though with varying trends depending on the zones. In the Southwest, production is rising steadily (7% per year) and accounts for around 30% of national production, as opposed to 10% in the 1950s. Production in the Centre and South has declined since the beginning of the 1970s, with different trends depending on the departments: downward in Mbam, stable in Lékié, but with sharp declines in Mefou, Nyong and So'o, and in Dja and Lobo.

The stands comprise of approximately 50% of plantations over 30 years old (especially in the Centre and the South, where 40% of the stands were planted before 1950). The production units are small and yields are low, as the farming systems are mostly extensive.

Up to the end of the 1980s, the cocoa commodity chain was totally run by the State. Various public and parapublic organisations provided upstream production services (subsidised input supplies, plantation treatments, technical supervision and training, creating tracks, granting credit). Lastly, a cooperative system under close State supervision provided the interface between producers and downstream or upstream bodies. The reform launched in 1989 with financial adjustments, followed by the introduction of a co-management system over the 1990/1994 period (founding of CICC and of ONCC), led in 1995 to almost complete liberalisation of the commodity chains, notably with the total opening up of marketing operations. The commodity chain management bodies (ONCPB, SODECAO, FONADER, COOP/MUT) either disappeared or their activities were severely curtailed.

In 2001, a survey was conducted among a limited number of cocoa producers in central Cameroon, in order to gain a clearer picture of the planting material grown and ascertain what the producers thought of it. This study also made it possible to identify cultural practices and planting prospects at a time when cocoa prices were at their lowest (376 CFA F/kg).

Material and methods

The survey was conducted over six weeks by a single interviewer in central Cameroon, involving approximately fifty farmers chosen at random in different areas:

- around Yaounde: in this zone, cacao competes with market garden crops, due to the proximity of the capital. In Lékié, (the villages of Nponsolo, Tallai and Akak), Nyong and Mfoumou, and Nyong and So'o, south of Yaounde,
- south of Yaounde: in Mvila department, in areas further away from the city,
- in Mbam and Kim, a cleared forest zone. The farms there are larger and better maintained. The survey was conducted around Talba, where the farmers have been settled for several decades. Available areas are becoming scarce, but the farmers remain enterprising.

The questionnaire submitted to the producers was broken down into three sections:

– description of the cocoa farm,
– cultural practices and planting material used,
– prospects for further planting by the farmers.

Results

1. The role of cocoa on farms

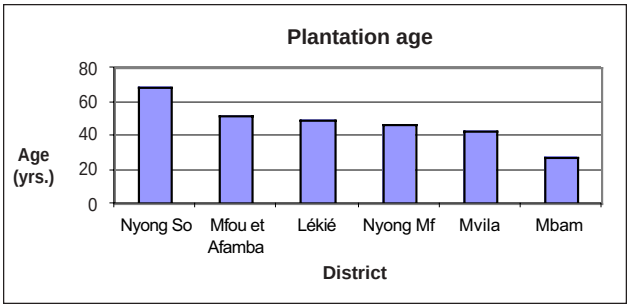
Cocoa provided the main income of the farmers. The average area planted in cacao was 6.5 ha, accounting for 22% of the area of the farm (29.5 ha on average), with the other crops occupying 4 ha, and forest reserves and fallow 19 ha. The share of land given over to cacao varied depending on the regions: 10 ha in Mbam, 2 to 3 ha in Lekié, Mfou Afamba, and Nyong and Mfoumou. Conversely, the land reserves per farm were much larger in Nyong Mfoumou (68 ha), and Nyong and So'o (38 ha) than in Lékié and Mbam (under 5 ha).

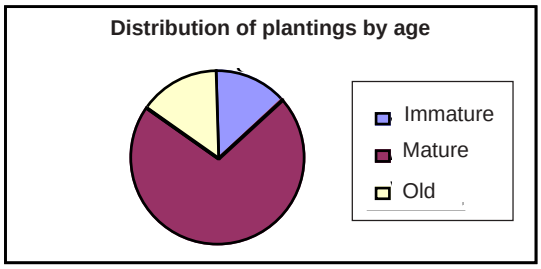
2. Cacao plantings

2.1. Age of the cacao stands

The cacao plots were generally old (50 years on average), most were planted between 1941 and 1979. It was therefore rare for the current farmers, at 48 years old on average, to have set up the plantation themselves.

Figure 1: Age of the first plantations depending on the region





Within plantations, 71.5% of the cacao trees were between 5 and 30 years old (mature), whilst only 15% of the trees were over 30 years (old) and 17.5% were under 5 years (immature). There had therefore been a substantial rejuvenation of the trees within plantings, which was partly explained by regular replacement of old trees that were no longer productive or dead.

The plantations had usually been established on cleared forest (96%) rarely on fallow or old coffee plantations (4 %), and never with former cacao plantings.

2.2. Crop management sequences

The average planting density for mature trees was 1,200 trees per hectare. However, the farmers used high densities when setting up young plantings, varying from 1,700 to 2,500 trees per hectare.

Seventy-five percent of the farmers maintained slight permanent shade, 20% dense shade, and 5% grew their cacao trees without shade. There were two types of shade, the first primarily consisting of medium sized fruit trees, such as mango, avocado, bush butter or palms and involving 31% of the cacao plantings, the second consisted of large native forest trees, which involved around 11% of the cacao plantings. The most frequent situation was a combination of the two (58%). Nitrogen fixing trees were very rarely used.

Production losses due to pest and disease attacks (mainly pod rot caused by *Phytophthora megakarya*) were estimated by producers at 50%, i.e. equivalent to 137 kg of cocoa per hectare. Eighty percent of them said that they applied 3 to 5 chemical treatments per year against pod rot (78% used Ridomil, and 64% contact products), but only 5% proceeded with a sanitary harvest each year. No farmer carried out maintenance pruning. Forty-two percent of the farmers carried out two chemical treatments per year, on average, against capsids, usually during the dry season.

Average annual yields per farm were approximately one tonne. Average commercial cocoa yields were 177 kg/hectare. This varied from 140 kg to 700 kg depending on the farm. The type of shade had a significant effect on yields: plots under slight shade from fruit trees were more productive (350 kg/ha) than plots under dense shade from native trees (180 kg/ha) or a combination of the two (100 kg/ha).

Fifty-six percent of the farmers carried out regular replacement planting in their plantation and sometimes proceeded with limited extensions around existing plots. On average, 200 plants were prepared each year, 60% of which were reared in the nursery, usually without polybags, and 40% by direct sowing.

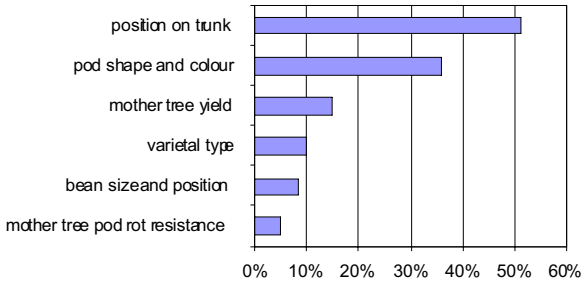
3. The varieties grown

Eighty-two percent of the cacao trees were of local origin (called "German Cocoa" of the Trinitario and Amelonado type), and only 18% came from hybrid type material disseminated by SODECAO and IRAD or were taken from neighbouring plantings (open-pollinated progenies). The distribution of controlled hybrid varieties had considerably slowed down at the time of the survey due to the withdrawal of SODECAO, and the difficulties encountered by PSCC over several years: only 6% of the producers declared having procured selected planting material from IRAD or from PSCC.

A majority of the farmers preferred local varieties due to their grouped production, longevity and lower susceptibility to pod rot. However, hybrids were appreciated for their precocity and yield, but criticised for their limited life span and their greater susceptibility to pod rot. Fifty-seven percent of the farmers were in favour of mixing both types of varieties to benefit from the different advantages and reduce risks.

Producers chose seeds of endogenous origin according to criteria that were not always linked to the individual genetic value of the mother trees (though they considered the old varieties to be more resistant on the whole), but more often to the ability of the seeds to produce well-developed plants.

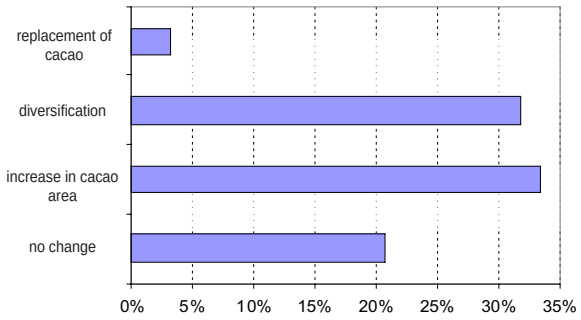
Figure 2: Criteria for the choice of endogenous seeds used by farmers



4. Planting dynamics and planting material requirements

The producers had planted virtually no new plots since 1995 although they all still had forest available. However, they proceeded regularly with replacement planting, and with extensions usually limited to the periphery of existing plots: an average of 200 young cacao trees were planted in this way each year, whilst they declared that they lost approximately a hundred trees per year. However, the dominant position accorded to cacao by the farmers in their farming systems remained stable: despite a tendency towards diversification, few of the farmers were considering replacing cacao by another crop in the future.

Figure 3: Future prospects for cacao



The farmers considering extensions estimated their annual requirements at 2,900 plants on average. They expected to plant on cleared forest (62%) especially in Mbam, on fallow around existing plots (19%) or on old cacao plantings (19%), especially in Lekié.

The demand of the farmers for selected hybrid varieties was very small. It reflected the limited confidence of farmers in the varieties distributed; instead the farmers intended to use local endogenous material for extensions or new plantings in 51% of cases. Moreover, they were no longer ready to invest in seeds while the price of cocoa remained so low.

However, in the event that they could be convinced of the merits of improved varieties whose precocity, yields, and particularly their level of rot resistance had been proved, they would be prepared to pay for such varieties, suggesting for example that the price of seeds be deducted at the time of harvest sales. Fifty-five per cent of them preferred to buy plants that had already been reared in the nursery as that saved time and guaranteed a vigorous plant that was easy to establish in the field. The others were more in favour of buying pods, due to the lower cost and ease of transport, but often expected to be provided with polybags and technical advice. The average price the farmers were prepared to pay for pods was estimated at 49 CFA F, and 84 CFA F for seedlings.

The majority of the farmers were keen for seedling supply organised at the village level. They would be prepared to manage a collective nursery themselves and benefit from mutual assistance in transporting seedlings to the plots. For organising such a collective nursery, 29% had confidence in the GIC (cooperative group), 18% in the village chief, 16% in SODECAO and 10% in IRAD.

Conclusion

This survey confirmed the preponderant role that cacao growing still plays for farmers in the central region of Cameroon in a rather difficult period following State withdrawal from the commodity chain, and with the low price paid for cocoa over several seasons. Not only did the farmers not intend to replace their cacao plantings with another crop, but they regularly proceeded with replacement planting and even envisaged extending their plantings in the short term. However, yields remained low, due to the number of trees over 40 years old, but especially due to a lack of control measures for mainly black pod, and lack of maintenance pruning. Most of the cacao trees being grown were of local origin, or derived from endogenous seeds from plantations.

A degree of farmer mistrust in the distributed hybrid varieties exists. The level of resistance to pod rot was often criticised, and the limited profitability of cacao growing, due to low cocoa prices, has led to the generalised use of endogenous seeds taken from smallholdings. These seeds were usually harvested from local material of Amelonado or Trinitario origin, which were replanted. This situation therefore slowed down the rate at which the traditional varieties were replaced by selected varieties. The main requirement for new selected varieties is that they have better resistance to pod rot (*P. megakarya*). Research to develop such new varieties is currently being carried out at IRAD. If the farmers are convinced of the superiority of new varieties, they would be ready to buy them as pods, or preferably, in ready-to-plant seedling form. For this to occur, they are awaiting a better distribution system and greater proximity of seedling production or distribution centres, along with acceptance of grouped orders by villages or cooperatives. In order to meet these expectations and pass the genetic gains achieved by research on to the farmers, the seed production and distribution system in Cameroon needs to be remodelled and, more especially, decentralised.

Accelerated Rate of Bud Sprouting on the Cacao Growth Mutant Root-stock

Y. Efron, E. Tade and P. Epaina

Introduction

The Cocoa and Coconut Research Institute in Papua New Guinea is experimenting with grafting of orthotropic buds. The objective is to produce clonal planting material with the orthotropic growth habit, similar to a hybrid cacao tree, with which the local farmers are familiar. Several practical problems for large-scale production of orthotropic clonal seedlings were identified. The factors affecting jorquette height were described in a previous INGENIC Newsletter issue (Efron *et al.*, 2000). Currently, bud dormancy that causes late and uneven sprouting of the grafted buds is the main obstacle to commercialisation of orthotropic buddings.

A cacao growth mutant with dwarfing effect as rootstock was recently identified (Efron *et al.*, 2002). The mutant (MJ 12-226) had a peculiar growth habit producing multiple orthotropic stems, and strong branching habit of fan branches. It was speculated that the mutation affected the quantity or the balance of growth hormones.

Orthotropic bud dormancy is probably caused by apical dominance, which is related to plant hormones. The phenotype of the mutant suggested a weak apical dominance. The aim of the following study was to test the hypothesis that buds grafted onto the mutant as rootstock would sprout faster and more uniformly.

Materials and Methods

The effect of the growth mutant on bud sprouting was tested in two different experiments:

Experiment 1

Root-stocks:

- Two week-old, normal root-stock obtained from open pollinated pods of clones with big beans (RST),
- Two month-old, normal segregants from open pollinated pods harvested from the mutant clone MJ 12-226 (N),
- Two month-old mutant segregants from open pollinated pods harvested from the mutant clone MJ 12-226 (M).

Scions:

- Plagiotropic (fan branch) buds from the clone 21-4-8,
- Plagiotropic buds from the mutant clone MJ 12-226,
- Plagiotropic buds from the clone 33-15/1,
- Orthotropic buds from the clone 33-15/1.

Patch (green) budding was done in all possible root-stock/scions combinations, 30 buddings per combination. Budding tape was removed 14 days after budding, and sprouting (initial bud growth) was recorded at two-day intervals thereafter. Strike rate (green patch) was counted during budding tape removal.

Experiment 2

Root-stocks: Normal (N) and mutant (M) segregants obtained from controlled hand pollination of the mutant MJ 12-226 (female) and several other clones as males.

Scions: Plagiotropic and orthotropic buds of the clones 17-3/1, 33-15/1 and 37-13/1.

Buddings and recordings were done as in experiment 1 on 60 seedlings per root-stock/scion combination.

Results

Experiment 1:

Sprouting started at day 16, two days after removal of the budding tape. Plagiotropic buds started to sprout earlier and at a faster rate than orthotropic buds (Figure 1a). Among the clones, 33-15/1 started to sprout later and at a slower rate than 21-4-8 on both the normal (Figure 1b) and the mutant (Figure 1c) root-stocks. However, the most noticeable effect on the rate of sprouting was obtained with the mutant genotype either as a scion or, in particular, as a root-stock. The fastest sprouting rate was obtained with plagiotropic buds of the mutant budded on the mutant root-stock, whereby at day 20 more than 90% of the buds had sprouted (Figure 1e). The accelerated sprouting on the mutant root-stock was obvious in both plagiotropic budding of the clone 21-4-8 up to day 24 (Figure 1f) and orthotropic budding of the clone 33-15/1 up to day 32 (Figure 1d).

Experiment 2:

The results of the second experiment were similar to those obtained previously. Budding strike rate was always more than 90% except for the orthotropic buds of 37-13/1 on the mutant root-stock (Table 1). The plagiotropic buds of the three clones sprouted earlier and faster than the orthotropic buds, reaching about a 90% sprouting rate 32 days after budding. Sprouting of the orthotropic buds was slower and more gradual. More than 15% of the buds were still dormant 40 days after budding.

The sprouting rate of both the plagiotropic and orthotropic buds was always earlier and faster on the mutant than the normal root-stocks during the first month after budding (Figure 2). The difference between the two root-stocks was highest at day 20 for the plagiotropic buds (31-38%) and on day 24 for orthotropic buds of 17-3/1 (37%) and 33-15/1 (35%). Orthotropic buds of 37-13/1 sprouted more slowly on both root-stocks with a maximum difference of 22% at day 28 between the mutant and the normal root-stocks.

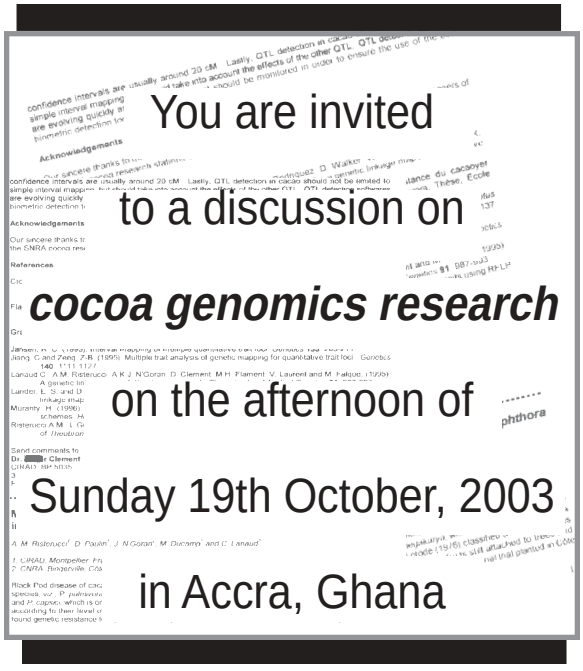
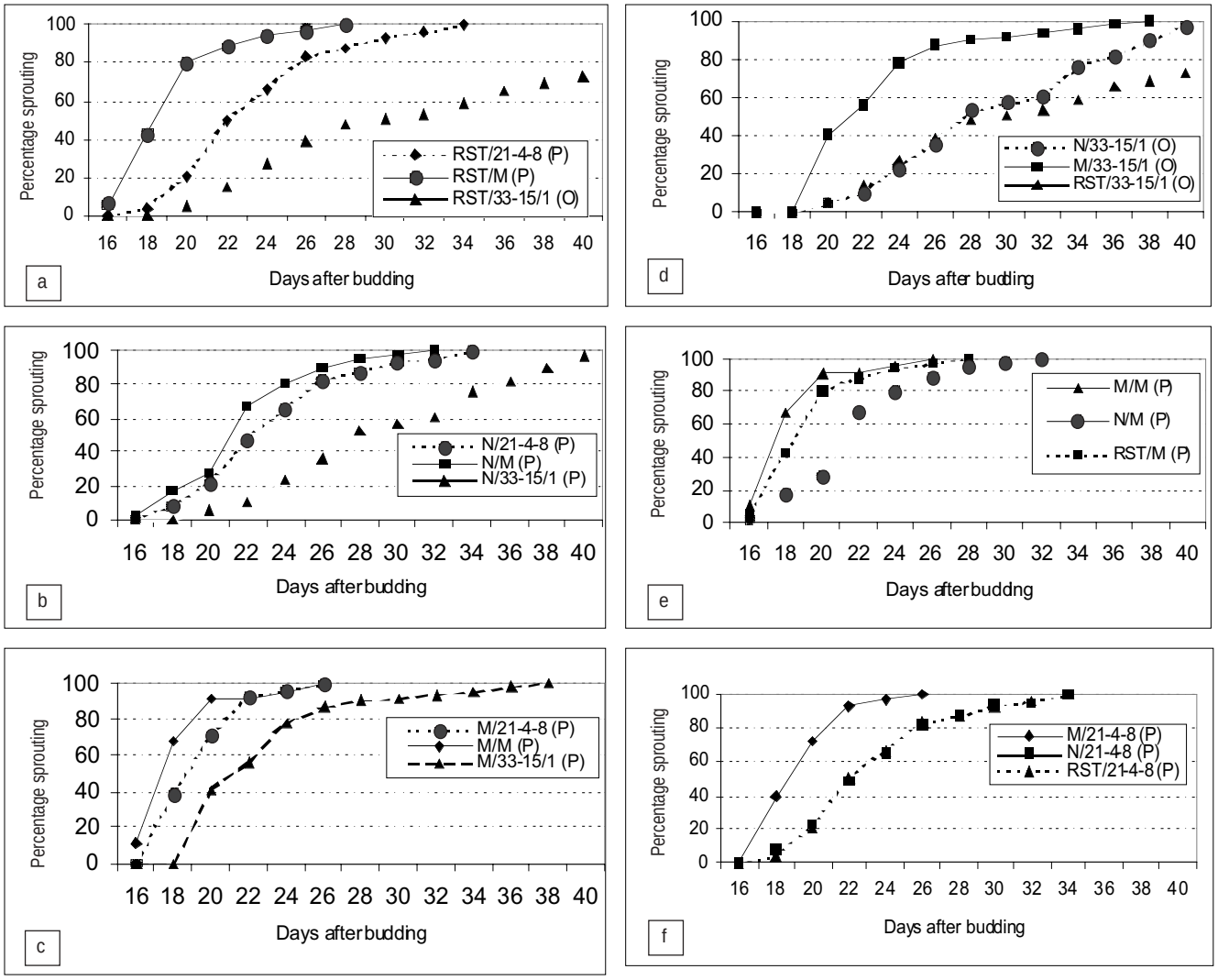


Table 1: Progressive sprouting (%) of plagiotropic and orthotropic buds of the clones 17-3/1, 33-15/1 and 37-13/1 budded on normal and mutant root-stocks

Clone	Bud type	Root-stock	Strike rate (%)	Percent sprouting days after budding						
				16	20	24	28	32	36	40
17-3/1	P	N	100	27	45	65	83	90	97	97
		M	100	47	76	84	91	97	98	98
		D	–	20	31	19	8	7	1	1
	O	N	100	7	12	20	40	60	80	85
		M	100	23	40	57	67	68	80	85
		D		17	28	37	27	0	0	0
33-15/1	P	N	100	8	30	53	82	97	97	97
		M	98	38	68	75	82	88	92	92
		D		30	38	22	0	-9	-5	-5
	O	N	93	0	5	13	28	61	66	72
		M	92	7	20	48	60	65	82	83
		D		7	15	35	32	4	26	11
37-13/1	P	N	100	10	20	47	62	85	85	85
		M	100	22	58	72	77	93	95	95
		D		12	38	25	15	8	10	10
	O	N	98	3	8	18	30	65	67	72
		M	77	8	13	30	52	63	67	68
		D		5	5	12	22	-2	0	-4

Figure 2: Relative sprouting and initial growth of orthotropic buds of the clone 17-3/1 on a mutant (front) and normal (back) root-stock

Figure 1: Progressive sprouting rates (%) of plagiotropic and orthotropic buds in various root-stock and scion combinations where P = plagiotropic, O = orthotropic, N = normal segregants, M = mutant segregants, RST = common root-stock



Discussion

The hypothesis that buds grafted onto the growth mutant MJ 12-226 as a root-stock would sprout faster than on a normal root-stock was fully confirmed. Both plagiotropic and orthotropic buds showed faster rates of sprouting up to about one month after budding. Similarly, buds of the mutant that were used as scion also sprouted faster than buds of other clones.

It is not yet known if the dwarfing root-stock would be of commercial value. This issue is presently under investigation. However, the main interest in the effect of the mutant on sprouting was related to orthotropic budding. Assuming that bud dormancy and sprouting are affected by hormones, a comparative study between the normal and the mutant root-stocks can provide a clue to the type and concentration of hormone(s) that can break down dormancy and induce faster sprouting. Based on this information, a technology to induce faster and more uniform sprouting of orthotropic buds by artificial application of plant hormones of the right type and concentration may be developed.

Differences were also observed in the sprouting rates of different clones. It suggests that the sprouting rate is probably under genetic control and therefore improved sprouting rate can be achieved by breeding.

Acknowledgement

The authors wish to express their gratitude to Mars Confectionery International for supporting part of the research described above.

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Inheritance of a Cacao Growth Mutant, MJ 12-226: a Possible Interaction between Nuclear and Cytoplasmic Genes

Y. Efron, D. Nideson, P. Epaina and E. Tade

Introduction

A cacao growth mutant with dwarfing effect as root-stock was identified at the Cocoa and Coconut Research Institute in Papua New Guinea (Efron *et al.*, 2002). The effect of the mutant as root-stock was tested using mutant and normal segregants obtained from open-pollinated pods harvested from the mutant clone MJ12-226. At the same time, seedlings derived from a cross between KEE 42 as female and the mutant MJ12-226 as male were all normal. The simplest possible explanation at that time was that the mutant phenotype was controlled by a recessive allele. The appearance of mutant segregants in seeds from open-pollinated pods was explained by possible partial self-pollination due to mixed self and foreign pollen carried by the pollinating insects. However, further studies showed that the ratios between the normal and dwarf segregants were always close to 1:1. This raised a question about the hypothesis of a recessive allele because it was difficult to accept that the pollinating insects always carry equal proportions of self and foreign pollen.

The following article describes the results obtained from controlled reciprocal crosses between the mutant MJ12-226 and several other clones.

Materials and Methods

Controlled hand pollinations were done between the mutant MJ12-226 as female and four normal clones – OTC-1, Matina 1-9, KA2-101 and EET 308 - as males. Reciprocal crosses were done between KA2-101 and EET 308 as females and the mutant MJ12-226 as male. The normal clones were selected because of their dark red flush.

When the pods matured, seeds were planted in planting bags in the nursery, and their phenotype was determined two months after planting when the two phenotypes were clearly distinguishable. Seeds from open-pollinated pods of the mutant clone MJ12-226 were also included.

Results

All the crosses with the mutant clone MJ12-226 as female showed a significant fit to a 1:1 ratio between the normal and mutant phenotype, similar to the ratio obtained from the open pollinated pods (Table 1, Figure 1). When the mutant clone was used as the male, all the seedlings showed a normal phenotype except for 10 out of the 585 seedlings tested that showed a mutant phenotype.

Reciprocal differences were also observed in the relative growth rate of the normal phenotypes (Figure 2). The average height of 10 normal seedlings from the cross EET 308 x MJ12-226 was significantly higher by 24.1% than the average height of 10 normal seedlings from the reciprocal cross, MJ12-226 x EET 308 (34.0 cm and 27.4 cm, respectively).

Figure 1: Segregating mutant and normal seedlings from the cross MJ12-226 x KA2-101 (front). The seedlings at the back are all normal from the reciprocal cross KA2-101 x MJ12-226

Figure 2: Normal seedlings from the cross MJ12-226 x EET 308 (left) and the reciprocal cross EET 308 x MJ12-226 (right)

Table 1: Number of normal and mutant segregants and goodness of fit to a 1:1 ratio in reciprocal crosses between the cacao growth mutant MJ12-226 and several normal clones

Cross	Number of seedlings		Total	Chi-square value	P
	Normal	Mutant		(λ)	
MJ12-226 O.P	416	392	808	0.66	0.25 – 0.50
MJ12-226 x OTC-1	136	154	290	1.52	0.10 – 0.25
MJ12-226 x Matina 1-9	114	98	212	2.50	0.10 – 0.25
MJ12-226 x KA2-101	117	135	250	0.90	0.75 – 0.90
MJ12-226 x EET 308	363	347	710	0.42	0.75 – 0.90
Total	**1146**	**1126**	**2272**	**0.08**	**0.75 – 0.90**
KEE 42 x MJ12-226 [1]	212	0	212		
KA2-101 x MJ12-226	102	4	106		
EET 308 x MJ12-226	261	6	267		
Total	**575**	**10**	**585**		

[1] Results from the previous study

Discussion

A 1:1 ratio is usually obtained in test crosses between heterozygous and homozygous recessive genotypes. The uniformity of 1:1 segregation between normal and mutant segregants, when the mutant clones were used as female parent, indicates that the previous assumption that the mutant phenotype is due to a recessive allele was incorrect. A possible alternative model is that the dwarf mutant phenotype is due to a dominant allele (DM_1), and the clone MJ12-226 is heterozygous DM_1dm_1. The genotype of the normal clone is homozygous recessive, dm_1dm_1, and the progenies segregate in a 1:1 ratio to DM_1dm_1 (mutant) and dm_1dm_1 (normal). The data obtained fit this model well.

An interaction between nuclear and cytoplasmic genes may explain the reciprocal differences. Accordingly, the cytoplasm of the mutant MJ12-226 (n) is different from the normal cytoplasm (N) and the DM_1 allele can express the mutant phenotype only in the n cytoplasm as follows:

Cytoplasm	Genotype	Phenotype	Seedling height
N	DM_1dm_1	normal	normal
N	dm_1dm_1	normal	normal
n	DM_1dm_1	mutant	dwarf
n	dm_1dm_1	normal	intermediate

The difference observed in seedling height of the reciprocal crosses supports the hypothesis of different cytoplasms and hints that it is possibly due to mitochondrial genes that are involved in the mechanism of energy production. A transmission of male mitochondria (Motamayor, pers. comm.) can explain the appearance of few mutant phenotypes when MJ12-226 was used as male parent. Similar examples of interaction between nuclear and cytoplasmic genes are well known, *e.g.*, male sterility in maize and sorghum.

The reciprocal differences may also be explained by an alternative hypothesis. When the mutant clone (DM_1dm_1) was used as male parent, there were two types of pollen grains, DM_1 and dm_1. It is possible that the DM_1 pollen grains were either not viable or their pollen tubes grew more slowly than those of the dm_1 pollen grains. Therefore, most of the ovules were pollinated by dm_1 pollen grains. This hypothesis explains the very low frequency of mutant phenotypes obtained when the mutant clone was used as a male parent. However, it does not explain the reciprocal differences in seedling growth. The reciprocal differences in seedling growth may possibly be attributed to the bigger seeds of EET 308 rather than the seeds of MJ12-226. Additional studies are required to differentiate between the two models.

Currently, only 50% of the seedlings show the mutant phenotype. They can be identified as mutant only about 2 months after planting. If the dwarfing effect of the mutant would be of value to obtain smaller trees with improved harvest index, it will be necessary to find a way, if possible, to increase the proportion of mutant segregants. Therefore, it is important to continue with the genetic studies. In particular, it is important to develop a homozygous DM_1DM1_1 genotype. If viable and productive, all its progenies should be of the dwarf type. The mutant clone itself is self-incompatible.

The reciprocal differences in the growth rate of the seedlings and the possible effect of the cytoplasm are of particular interest. If verified and the differences in growth rate persist with time, the use of the mutant cytoplasm may provide a way to reduce tree vigour of both hybrids and clones which are unrelated to the root-stock.

Acknowledgement

The authors wish to express their gratitude to Mars Confectionary International for supporting part of the research described above.

Reference

Efron, Y., Nideson, D., Epaina, P. and Faure, M. (2002). A cacao growth mutant with dwarfing effect as rootstock. *INGENIC Newsletter* **7**: 16-19.

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Differential Responses of Big, Intermediate and Small Size Cacao Clones to Increased Planting Density in Papua New Guinea

Y. Efron, P. Epaina, J. Marfu and S. Mombi

Introduction

The importance of root-stocks that control tree size of cacao was discussed by Purdy and Eskes (2002). The first example of a dwarfing cacao rootstock was described by Efron *et al.* (2002). However, reduced tree vigour can also be achieved by breeding and selection of clones, regardless of the root-stock, which is being used.

Smaller cacao trees would be of value only if they are planted at higher density. In the Cocoa and Coconut Research Institute (CCRI) of Papua New Guinea, the clones developed are usually divided into three size categories – big, intermediate and small. The results presented in the following article were obtained from multi-location testing of advanced clones in preparation for release of polyclonal varieties.

Material and methods

Twenty-nine Upper Amazonian x Trinitario derived hybrid clones and two Trinitario clones (K82 and KA2-101) were included. The clones were divided based on visual observation into big, intermediate and small size categories and tested in separate sub-trials at the same sites. The Trinitario clones were used as common controls for the three sub-trials. Each sub-trial was planted at two densities (625 and 1,000 trees/ha) in a split plot design with densities as main plots and clones as sub plots. There were four replications/density/clone with 12 trees/plot.

The trial, as described above, was planted at two locations:

- At Tavilo, East New Britain Province, a major cocoa producing area with fertile volcanic soil and moderate rainfall of about 2,500 mm. The trial was planted in a cleared cacao block under *Gliricidia* shade during 1995.
- Hawain, East Sepik Province, in the western part of the country with sandy loam soil and about the same amount of rainfall as in Tavilo. The trial was planted in 1996 on a newly cleared forest area under *Gliricidia* shade.

Dry bean yield was estimated by harvesting and counting the pods fortnightly. Pods and wet bean weights were counted and measured, respectively, several times during periods of peak harvest using all the pods harvested/plot. A uniform rate of 30% was used to convert wet to dry beans. The relative vigour of the trees was verified in Tavilo by measuring trunk circumference 20 cm above the ground in 2002, seven years after planting.

Results

Meaningful pod production started at both sites about 18 months after planting. The small clones were more precocious than the intermediate and big clones, as reflected by their higher yields during the first year of production in both sites (Table 1).

The average yield of all the clones in the three sub-trials was always higher at the density of 1,000 trees/ha than at 625 trees/ha (Table 1). However, the magnitude of the difference between the high and low densities varied according to the size of the clones.

The response to increased density was highest in the small clones with a total increase of 1693 kg/ha (25.0%) in Tavilo and 2022 kg/ha (41.1%) in Hawain. It was followed by the intermediate (10.8% and 29.6%, respectively) and the big clones (10.3% and 18.3%, respectively) at the two sites.

A comparison between the total yield of the small and big clones showed that at the higher density the yield of the small clones was higher in Tavilo by 923 kg/ha and in Hawain 543 kg/ha (Table 2). However, at the lower density, the yield of the small clones was lower by 62 kg/ha at Tavilo and 490 kg/ha at Hawain.

The magnitude of the difference between the two densities was also age dependant (Figure 1). In the first year of production, the yields at the high density were higher by 60-70% except for the small clones in Tavilo where the difference was 93.7% (Table 1). As the trees became older, the magnitude became smaller with a sharper decrease between year one and two. There were no significant clone x density interactions in any of the sub-trials at the two locations.

The average trunk circumference of the individual clones ranged from 31.5 cm for the clone 23-6/1 (small) to 44.9 cm for 38-8/2 (big). As groups of clones, the average trunk circumference was 41.6, 36.9 and 35.3 cm for the big, intermediate and small clones, respectively (Table 3). High density planting reduced the average trunk circumference by eight percent (approximately 3.0 cm). Usually, the measurements within treatments were very uniform, resulting in a low C.V. of 5.2%.

Table 1: The effect of planting density on the average yield of small, intermediate and big size clones in Tavilo and Hawain during 1997 to 2001

Average dry bean yield (kg/ha)									
Location	**Clone size**	**Density**	**1997**	**1998**	**1999**	**2000**	**2001**	**Total**	**D**
Tavilo	Small	High	707*	1480*	2942*	2053*	1286*	8468	1693
		Low	365	1061	2573	1711	1065	6775	
		H:L (%)	193.7	139.5	114.3	120.0	120.7	125.0	
	Intermediate	High	643*	1548*	2486	1871	1108*	7656	748
		Low	402	1404	2367	1735	1000	6908	
		H:L (%)	160.0	110.3	105.2	107.8	110.8	110.8	
	Big	High	323*	1267*	2663*	2163	1129	7545	708
		Low	202	1040	2377	2125	1093	6837	
		H:L (%)	160.0	121.8	112.0	101.8	103.3	110.3	
Hawain	Small	High		925*	2301*	1721*	1999*	6946	2022
		Low		538	1777	1251	1356	4922	
		H:L (%)		171.9	129.5	137.6	147.4	141.1	
	Intermediate	High		645*	2158*	1556*	1911*	6270	1431
		Low		395	1597	1308	1539	4839	
		H:L (%)		163.3	135.1	119.0	124.2	129.6	
	Big	High		274*	1590*	2320*	2219*	6403	991
		Low		166	1305	1908	2033	5412	
		H:L (%)		165.1	121.8	121.6	109.1	118.3	

High density, 1000 trees/ha
Low density, 625 trees/ha
H:L (%) = Relative yield at high density compared with low density (100%)
D = The difference (kg/ha) between the total yield at high density and low density.
* = Significantly (5%) higher yield at high density than low density.

Table 2: Total dry bean yield of small and big clones in high and low density at Tavilo and Hawain

Density	Total dry bean yield (kg/ha)					
	Tavilo			Hawain		
	Small	Big	D	Small	Big	D
High	8468	7545	923	6946	6403	543
Low	6775	6837	-62	4922	5412	-490

High density 1000 trees/ha
Low density 625 trees/ha
D – The difference between small and big clones

Table 3: Average trunk circumference of small, intermediate and big clones in two densities at Tavilo, seven years after planting

Clone size	Trunk circumference (cm)		
	Low density	High density	Average
Big	43.5 A (A)	39.6 A (B)	41.6 A
Intermediate	38.5 B (A)	35.3 B (B)	36.9 B
Small	36.4 C (A)	34.3 B (B)	35.3 C
Average	39.5 (A)	36.9 (B)	

Numbers showing the same letters are not statistically significant at the 5% level (Newman-Keul's test). The letters in brackets refer to the density effect within the size groups

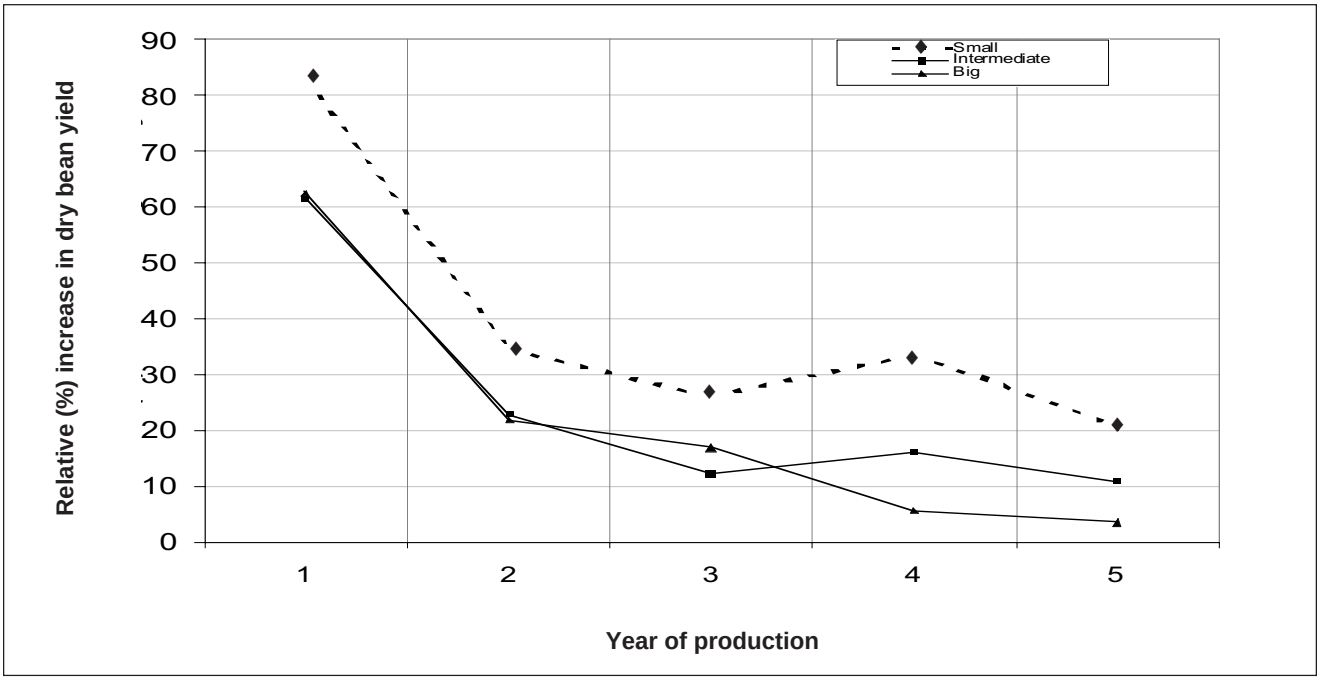

Figure 1: The average effect of tree age on the relative yield increase (%) at high density compared with low density of small, intermediate and big clones in Tavilo, ENB and Hawain, East Sepik

Discussion

Cacao genotypes growing under the same conditions may differ in their vegetative growth rate and potential tree size. Accordingly, the optimal planting density can be related to the relative vigour of the trees. Theoretically, assuming no competition between trees, the expected yield at the higher density was 1000:625 = 1.6 or 160% compared with the low density. These expected ratios were obtained during the first year of production in both sites except for the small clones in Tavilo where, for unknown reasons, the difference between the two densities was higher. At this age, the trees were relatively small, their canopy was not closed and therefore, they did not compete with each other. However, by the second year of production, the continued growth of the trees had closed the canopy between them and exposed them to interplant competition. As a result, the yield advantage at the high planting density was reduced during the second year of production. This trend continued at a milder rate from year 2 to year 5 (Figure 1). The effect of the interplant competition was related, as expected, to the vigour of the trees, smaller in the small clones and larger in the more vigorous trees. In Tavilo, the yields of the big clones in the 4th and 5th years were already very similar at the two densities.

Planting at higher density was very beneficial economically, particularly in the small clones. At Tavilo, the total yield difference was 1693 kg/ha. This is equivalent to an average of US$338.6 per year at US$1,000/tonne. High density planting incurs additional costs for establishment and management. This includes the cost of planting material, transport and planting. The additional cost for pruning and harvesting should also be considered. In total the average annual additional cost was estimated to be US$48.6 leaving an average annual net profit of US$290.0/ha.

Based on the results of the multi-location testing, CCRI released two poly-clonal varieties of small and big trees with four clones each early in 2003. A farmer may wonder which variety to choose. The results show that he should consider his plans for planting density. At a lower density, he should be advised to plant the bigger clones. However, at a higher density he should prefer to use the smaller clones. Moreover, the vigour of a tree is relative. It depends on the growing conditions, which should also be taken into consideration. In poor soils, it might be better to plant the more vigorous clones.

Conclusion

Cacao clones of different vigour can be developed by breeding. Smaller clones respond better to increased planting density. However, the vigour of a cacao tree is relative and depends on the growing conditions. A small clone that grows well in a good fertile soil with sufficient moisture may be too slow growing under poor growing conditions. Thus, the optimal planting density should be a function of both the potential vigour of the trees and the growing conditions.

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Differential Responses of Trinitario Cacao Clones to Attack by the Trunk Longicorn, *Glenea aluensis*

Y. Efron and P. Epaina

Introduction

Many kinds of longicorn beetle *(Coleoptera: Cerambycidae)* feed on cacao. The most common species in the Islands Region of Papua New Guinea (PNG) is *Glenea aluensis* (Figure 1). Longicorn larvae can cause extensive damage to the cacao tree by boring into the trunk (Figure 2) and, less frequently, main branches to feed on sapwood. A single larva can totally ring bark the base of the trunk causing the death of the tree.

Observations at the PNG Cocoa Research Institute (CCRI) have shown that cacao clones are usually more vulnerable to longicorn attack than hybrids, and that Trinitario clones are more severely affected than Upper Amazonian clones or Trinitario x Upper Amazonian derived clones.

Material and Methods

Two replicated Trinitario clone trials were planted in 1996 as part of a Trinitario population improvement programme. The clones were derived from selected progeny trees of diallel crosses between 10 Trinitario clones (Table 3). Unequal numbers of clones were selected from the different crosses. They were divided, based on the vigour of the mother trees, into two groups: big (Trial 146-B) and small (Trial 146-S), with 73 and 84 clones, respectively. The two trials were planted in 3 replications, 9 trees/replication under *Gliricidia sepium* shade. Four Trinitario clones, K82, KA2-101, KA2-106 and Rum Jungle 2 were used as common controls in the two trials. The first three clones were also used as parents for the original crosses. The Upper Amazonian clone, KEE 47, was also included as a control, but only in Trial 146-B.

The first signs of longicorn damage were observed in 2000. Three rounds of insecticide channel painting with a mixture of Dichlorovos (30 ml), Ridomil (15g), white oil (250 ml) and water (720 ml) at six-month intervals were used in an attempt to control the insect. However, the treatment was not very effective. By early 2002, the damage was very severe and many trees died.

Observations in blocks of the trials revealed a different degree of damage in the various plots (Figure 3). This promoted a detailed survey of individual trees using the following scores:

0 = unaffected tree
1 = signs of previous longicorn tunnelling in the trunk, but the tree canopy was not affected
3 = active longicorn tunnelling and signs of canopy stress
5 = a dead tree

The scores of all the nine trees in the plots were added to give a longicorn damage score for the plot that could range from 0-45. It should be indicated that the death of some trees could be due to other reasons, but the major cause was taken as that of longicorn.

Figure 1: An adult Trunk Longicorn beetle, *Glenea alluensis*

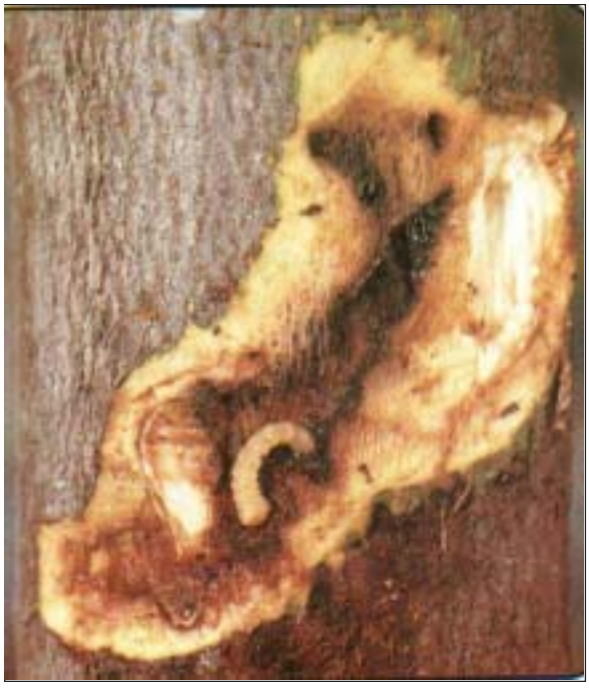

Figure 2: Damage to a cacao tree due to ring barking by a Trunk Longicorn larva

Figure 3: Longicorn damaged tree surrounded by unaffected trees

Results

Considering the nature of field infestation by an insect, the scores obtained in the three replications were relatively uniform, but this was not always the case (Table 1).

Table 1: Uniformity of longicorn damage scores of several Trinitario clones in three replications

Clone	Longicorn damage score			
	Rep I	Rep II	Rep III	Average
T78-3-4	3	9	6	6.0
T310-3-16	9	13	8	10.0
T18-1-10	40	31	42	37.7
T18-1-7	40	25	45	36.7
T78-2-11	22	9	8	13.0
T49-3-9	30	9	31	23.3
T25-1-7	35	33	15	27.7

A comparison between the common control clones in the two trials (excluding KEE 47) has shown very similar results (Table 2). In both, KA2-101 had significantly lower scores than K82, Rum Jungle 2 and KA2-106. The Upper Amazonian clone, KEE 47, in Trial 146-B had a lower score similar to that of KA2-101.

Table 2: Average longicorn damage scores of the control clones in Trials 146-B and 146-S

Clone	Average Longicorn damage Score		
	146-B	146-S	Average
K82	31.7 A	29.3 A	30.5
Rum Jungle 2	23.3 AB	29.7 A	26.5
KA2-106	21.3 AB	23.0 A	22.1
KEE 47	12.3 BC		
KA2-101	7.0 C	11.3 B	9.1
Average	**20.8**	**23.3**	**22.0**
C.V. (%)	**30.5**	**23.5**	

Means sharing the same letter are not significantly different at the 5 % level (Newman- Keuls's test)

The average longicorn damage score of the tested clones ranged from 6.0 to 40.0 and 6.3 to 38.0 in trials 146-B and 146-S, respectively. The ANOVA has shown highly significant differences between the clones in both trials. The distribution of the damage scores for all the clones in both trials was approximately normal (Figure 4), whereby 24.2% of the clones had an average score below 15.

The 10 parental Trinitario clones differed as donors for resistance to attack by the longicorn (Table 3). The crosses with K20 had the lowest mean score of 16.4 followed by KA2-101 (18.0). The clones derived from the cross between these two clones had the lowest

average score of 6.0. The clones 58/24 and K82 were donors for the highest mean scores of 24.5 and 23.0, respectively. The lowest average scores were obtained from the crosses K20 x KA2-101 (6.0); K13 x K6 (9.5) and K20 x K13 (9.6). The highest average damage scores were obtained from KT140 x KA5-201 (31.8); KT140 x 58/24 (30.0) and 58/24 X KA2-101 (29.7). Three of the parental clones, K82, KA2-106 and KA2-101, were used also as control clones. Their scores as clones (Table 1) were reflected in the scores of relative damage of the clones derived from them as parents.

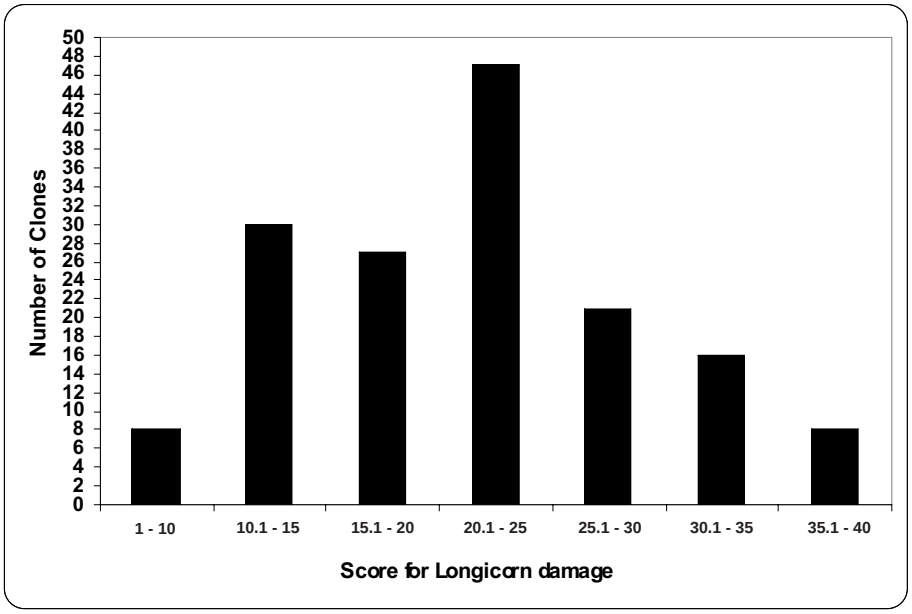

Figure 4: Frequency distribution of damage to Trinitario cacao clones by trunk Longicorn, *Glennea Aluensis*

Table 3: Mean longicorn damage scores of clones derived from diallel crosses between 10 Trinitario clones

Clone	KA2-101	K13	K6	KA2-106	K23	KT146	KA5-201	K82	58/24	Mean	No. Clones
K20	6.0	9.6	21.0	20.4	23.1	12.0	21.7	20.5	13.7	16.4	21
KA2-101		24.4	15.7	12.0	16.0	23.1	13.7	21.5	29.7	18.0	23
K13			9.5	n.t	n.t	19.0	21.5	28.7	n.t	18.8	23
K6				27.2	22.4	22.0	n.t	18.8	23.3	20.0	30
KA2-106					19.7	19.5	21.5	21.2	21.3	20.4	35
K23						19.5	19.1	18.7	25.7	20.5	38
KT140							31.8	26.1	30.0	22.6	37
KA5-201								26.6	27.3	22.9	27
K82									25.3	23.0	41
58/24										24.5	28

n.t – Clones from the cross were not included in the trials.

Discussion

The results presented show that there are significant differences between Trinitario clones in the degree of damage caused to them by the longicorn. These differences are probably under genetic control. However, it is not known if they are due to preference by the longicorn female to choose the trees for laying eggs, bark and trunk physical characteristics of the clones or intrinsic biochemical factors. Potentially, these differences can be explored by breeding to develop cacao genotypes with tolerance to longicorn. However, it requires developing an appropriate screening methodology, which may be extremely difficult to achieve.

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USDA Cacao DNA Fingerprinting Ring Test: Results from Penn State University

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Introduction

In fall of 2000, at the INGENIC meeting in Malaysia, a presentation was made by Dr. James Saunders of the USDA reporting on the development of a programme to fingerprint most if not all cacao germplasm in the international collections using microsatellite markers. Microsatellite markers are small repetitive DNA sequences dispersed in genomes (Tautz, 1989). The lengths of these elements are hyper-variable and thus are highly polymorphic, making them ideal in genomic fingerprinting applications (Devey *et al.*, 2002; Rahman and Rajora, 2002; Testolin *et al.*, 2000). It was generally agreed that a cacao fingerprint database would be useful in establishing the genetic diversity of the collections, in understanding the relatedness between clones, in evaluating labelling consistency and mistakes, and in validation of the identities of clones that have been transferred between germplasm collections.

A discussion followed as to the adaptability of the method to different laboratories, its reproducibility and the ability to share data across platforms. An agreement was made to test the established protocols in several participating laboratories to validate the reproducibility of the method and establish agreed upon, international standards for cacao genomic fingerprinting. In November of 2000, Dr. David Butler distributed leaf samples from eight cacao accessions and in January of 2001, Dr. Saunders distributed sequences of 15 microsatellite primers chosen from the CIRAD collection as optimal for the test (Lanaud *et al.*, 1999). The results of the testing done with these materials at Penn State University are presented here. It is hoped that other participating laboratories can use these data to compare with their own, and eventually all the data will be combined into one summary document.

Cacao plant materials and DNA extraction

The Cocoa Research Unit in Trinidad provided all plant materials. The genotypes tested were PA 30 T1, LX 31, PA 30 T10, GU 114P, PA 30 T5, GS 4/4A, LCTEEN 68-1, and IMC 47. DNA was extracted using the Qiagen DNeasy DNA extraction kit and the recommended manufacturer's protocol. Once quantified, the DNA samples were stored at a concentration of 10ng/μL at 4 °C.

Primers

Fifteen fluorescent microsatellite primers were obtained from the US Department of Agriculture: mTcCIR7, mTcCir18, mTcCIR40, mTcCIR33, mTcCIR1, mTcCIR60, mTcCIR22, mTcCIR24, mTcCIR15, mTcCIR11, mTcCIR12, mTcCIR26, mTcCIR37, mTcCIR6, mTcCIR8. The first six primers listed had annealing temperatures of 51 °C, while the remaining nine had annealing temperatures of 46 °C. The sequences of individual primers may be found in Lanaud *et al.*, (1999).

PCR and Electrophoresis

PCR was carried out in 25μL total volume containing the following final concentrations: 1x GeneChoice Reaction Buffer (PGC Scientifics; 100 mM Tris-HCl pH 8.5, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100), 1 μM dNTP, 300 nM of both forward and reverse primer, 0.5u *Taq* DNA polymerase, 30 ng of DNA. The reactions were incubated in a Perkin-Elmer GeneAmp 9700

thermocycler for an initial melting step of 94 °C for 3 minutes, followed by 30 cycles of a melting step of 94 °C, and annealing step of 46 °C or 51 °C dependant on the primers used, and an elongation step of 51°C. Once the thirty cycles were complete, the reactions were incubated at 72 °C for 7 minutes and then stored for electrophoresis at 4 °C. Each PCR was replicated a total of three times.

PCR reactions were separated on an ABI 3100 automated DNA sequencing apparatus. Electrophoresis was carried out in 36 cm capillaries with the POP4 polymer at 60 °C at 15 kV for 1350 sec with an injection time of 22 seconds. Individual PCRs were separated with 0.5 μL of an internal size standard (X-Rhodamine MapMarker, Bio Ventures Inc.). This allowed accurate sizing of microsatellite bands by the Perkin Elmer Genotyper software.

Data Collection

The resulting microsatellite fragment sizes were recorded using the P-E Applied Biosystems Genotyper software. In most cases, more than a single or pair of band(s) were produced, as would be expected by co-dominant markers such as microsatellites. In a co-dominant case, it would be expected that any one genotype would have two bands present if it was heterozygous for that marker, or a single band if it was homozygous for that marker. Since we often scored more than two bands per primer pair and we did not have access to parental genotypes, the data were treated as being dominant in nature and scored in a binary fashion.

Eleven of the fifteen primers amplified clear DNA fragments as expected; the remaining four primers (mTcCIR40, mTcCIR33, mTcCIR12, and mTcCIR6) failed to prime amplification despite repeated attempts. For the eleven primers that did give good amplification, three replicate reactions were compared and the resulting fragments were regarded as being reproducible if they appeared in two out of the three replicates. Bands that appeared only once were regarded as being PCR artifacts and were discarded from the analysis. Next, we compared the fragments produced by each genotype, and discarded any monomorphic (non-informative bands) that appeared in all DNA samples. We also found some fragments that were within one base pair in size of one another. These fragments were considered to fall within the accuracy of the ABI 3100 and thus were considered as being the same, and were expressed as a range of sizes for further analysis.

Results and Discussion

The results were then summarised and presented in binary form (Table 1). From this table it can be seen that the eleven primers are more than sufficient to clearly distinguish among the eight genotypes tested. The primer pairs produced from two to twelve individual DNA fragments, which were both reproducible and polymorphic with the genotypes tested. A total of 61 such polymorphic markers were scored. However, 33 contained markers that were seen in only two of three amplifications, indicating some variability in the reproducible production of these fragments (Table 1, fragments indicated with a *). The lack of amplification with some of the primer pairs and the lack of reproducibility in the amplification of certain individual fragments highlight the need for development of unified, accepted international standards for genotype mapping. It will be interesting to see if the other participants in this ring test see similar variability in the amplification of the same fragments. Nonetheless, this method was shown to be very informative, in our hands, for molecular discrimination among the genotypes tested.

It is hoped, that by publishing these results, other laboratories involved in the cacao ring test will be able to compare their data to ours. If discrepancies are present then discussion should be made to resolve these issues so that a definitive protocol for microsatellite fingerprinting for global use can be defined. Our data can also be accessed at the Guiltinan Laboratory Website at The Pennsylvania State University (http://www.guiltinanlab.cas.psu.edu).

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		Accessions							
Primer	Fragment Size (bp)	1	2	3	4	5	6	7	8
mTcCIR7	118-120	0	1	1	0	1	1	1	1
mTcCIR7	132-133	1*	1	0	0	0	0	1	0
mTcCIR7	134	1*	0	1	1	1	1	1	1
mTcCIR7	138-139	0	1	0	0	0	0	1*	0
mTcCIR7	143-144	0	1*	0	0	0	0	1*	0
mTcCIR7	153-155	1*	1	1	1	0	0	0	1
mTcCIR7	156-158	1	1	1	0	1	1	0	1
mTcCIR7	162-163	0	0	0	0	0	1	0	0
mTcCIR7	168	0	0	0	1*	0	0	0	0
mTcCIR7	190	1*	0	1	1*	1	1	0	0
mTcCIR37	144	1*	0	0	0	0	1*	0	1
mTcCIR37	159-160	1*	0	0	0	0	0	0	1*
mTcCIR37	163	0	0	0	1	0	0	1	0
mTcCIR37	165	0	0	0	1*	0	0	1	1*
mTcCIR60	190-191	0	0	1*	0	1	1	0	1*
mTcCIR60	193	0	0	0	0	0	0	1	0
mTcCIR60	208-210	1	1	1	1*	1	1	0	1
mTcCIR60	211-213	1	1	1	1	0	0	1	1
mTcCIR60	221	0	1	0	1*	1*	1*	1*	1
mTcCIR1	127	0	0	1	1	0	0	0	0
mTcCIR1	129-131	1	1	1*	1	1	0	1	1*
mTcCIR1	143	0	1*	0	1	1	1	1*	1*
mTcCIR1	150	0	1	1	1	1	1*	1*	1*
mTcCIR11	129-130	0	1	1*	0	0	0	0	0
mTcCIR11	141	0	1	1*	1*	0	1*	0	0
mTcCIR11	253-254	1	1*	1*	0	1	1	1	0
mTcCIR11	272	0	0	0	0	0	1*	0	0
mTcCIR11	288	0	0	0	0	1	0	0	0
mTcCIR11	298-300	0	0	1	1	0	1*	1*	0
mTcCIR11	308	1	1	0	0	0	0	0	1
mTcCIR11	314	0	1*	0	1	0	0	1	1
mTcCIR11	324	0	0	1*	0	1	1*	0	1

		Accessions							
Primer	Fragment Size (bp)	1	2	3	4	5	6	7	8
mTcCIR18	320	0	1*	1*	0	0	0	0	0
mTcCIR18	330	0	0	1	0	0	0	0	0
mTcCIR18	332	0	0	0	0	1	1	0	0
mTcCIR18	334	0	0	1	0	1	0	0	0
mTcCIR18	342	1	1	0	1	0	0	1	1
mTcCIR18	344	1	1	0	1	0	0	1	1
mTcCIR18	354	0	0	0	0	0	1	0	0
mTcCIR22	120	1*	0	0	0	0	0	0	0
mTcCIR22	290	0	0	1	0	0	0	0	0
mTcCIR22	307-308	0	0	1	0	0	0	0	0
mTcCIR22	314	1*	1	0	0	1	1	1*	0
mTcCIR24	182	0	0	0	0	1*	0	0	0
mTcCIR8	239	0	1	0	0	0	1*	0	0
mTcCIR8	257-258	1*	1	0	0	0	0	0	1
mTcCIR8	264-266	1*	0	1	0	1	1	0	1
mTcCIR8	274-275	0	0	1	1	0	0	0	0
mTcCIR8	282-283	1	0	0	0	0	0	0	0
mTcCIR8	292	0	0	0	0	1	0	0	0
mTcCIR15	203	0	0	1	0	0	1	1*	0
mTcCIR15	221	0	0	1	0	0	0	1*	0
mTcCIR15	233	0	0	1	0	0	0	0	0
mTcCIR15	239	0	1	0	0	0	0	0	1
mTcCIR15	249-251	0	0	1	1	1*	0	1	1
mTcCIR15	284-285	1	0	0	0	0	0	0	0
mTcCIR15	300	1	0	0	0	0	0	0	0
mTcCIR26	292	0	0	0	1	0	0	0	0
mTcCIR26	294-296	0	1*	1	1	1	0	1*	1
mTcCIR26	301	1	1	0	0	0	1	1	1
mTcCIR26	303	0	0	1	0	0	0	0	0

Table 1: Binary tabular fingerprinting data for each of eight cacao genotypes using eleven microsatellite primers. Column 1 indicates the primer pairs used, column 2 is the sizes in base pairs of DNA fragments that were amplified by the respective primer. Cacao accessions 1-8 are as follows: PA 30 T1, PA 30 T10, LCTEEN 68-1, LX 31, IMC 47, GU 114P, GS 4/4A and PA 30 T5. 1 represents the presence of a band, while 0 represents the absence of the band. An indicates a fragment that only amplified in two out of three replicate experiment times. An example is that we would expect the genotype PA 30-T1 to have markers at 132-133 bp, 134 bp, 153-155 bp, 156-158 bp, and 190 bp, when amplified with the primer mTcCIR7

A New Approach to Screening for Resistance to Witches' Broom Disease in Cacao Breeding Programmes

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Scope

Screening for Witches' Broom resistance (causal agent: *Crinipellis perniciosa*) in *Theobroma cacao* L. in the past has been based on several inoculation methods, *viz.* seed inoculation (Holiday, 1957), terminal bud inoculation using agar blocks (Wheeler and Mepsted, 1988), spray inoculation of whole plants (Purdy *et al.*, 1997), callus inoculation (Fonseca and Wheeler, 1990) and indirect methods based on characteristics of spore germination in leaf extracts (Evans and Bastos, 1980) or phloem sap (Bastos and Albuquerque, 2000). However, none of these have fulfilled all of the criteria required of a screening tool – simplicity, repeatability, ability to quantitatively discriminate between the resistance levels of clones (precision), heritability and ability to correlate to field resistance (Zadoks, 1997).

Recently, an agar-droplet inoculation method (Surujdeo-Maharaj *et al.*, 2003), in which concentrated spores in a droplet of diluted agar is placed on the growing meristem, has shown promise. This inoculation method is different from the others in that a predetermined concentration of basidiospores is placed at a susceptible point so that 100% infection can be obtained on a repeatable basis in susceptible accessions, either clones or seedlings. This method shows promise to measure resistance to witches' broom disease more precisely.

The method involves placing a droplet (30 μl) of agar (0.3% agar) containing 350,000 basidiospores per ml on the apical meristem (at the flushing-2 stage) of 12-month-old seedlings or micrografted plants. The plants are incubated in polythene bags containing moist tissue paper for 60 hours at 25° C after which the plants are moved into a shade house (70% shade).

This inoculation method was then used in 14 clones and their progenies to study a number of resistance measures, *viz.*, proportion of plants infected, time taken to first evidence of swelling (incubation period), time taken to broom appearance (Figure 1), proportion of plants developing brooms, proportion of swellings that convert into brooms, stem swelling, broom base diameter, broom length and broom dry weight. These measures were compared based on precision (Coefficient of variation, or CV), ability to discriminate (Index of discrimination), repeatability, correlation to field resistance and heritability (strength of parent-offspring regression) to determine the best quantitative measure of resistance to witches' broom disease. The measures were also tested for their precision/ repeatability, accuracy and heritability as single plant estimates to determine their ability to be used effectively to select within segregating populations (Surujdeo-Maharaj *et al.*, in press).

Mechanism of resistance

The measures of resistance (in 14 clones and progenies) were subjected to correlation analysis to determine possible mechanisms of resistance to witches' broom disease. The results showed that the measures fell into two categories. The proportions of plants showing swelling, broom development and swellings that developed into brooms fell into one category. The other measures of resistance such as incubation period, broom base diameter, broom length and broom weight fell into a second category. Correlations were high between resistance measures within categories, but were moderate to low between categories. When the Scavina (SCA 6 and SCA 12) clones were removed from the analysis, there was no correlation between resistance measures of the two types of categories.

The results suggest that there are possibly two stages of resistance to witches' broom disease. The first stage involves success in establishing infection, while the second stage involves pathogen growth and colonisation, which elicits a host response that is measured by incubation period, stem swelling and broom size. The fact that the correlation between categories disappeared when SCA clones were removed suggests that both mechanisms may co-exist in the SCA clones.

Herein lies the strength of the agar-droplet inoculation method. Since the first barrier is breached in most clones (100 % infection obtained with all clones except SCAs and IMCs), it provides a more repeatable measure of resistance at the second stage, which is the severity of symptoms elicited by pathogen growth. This also makes the method more repeatable.

Measures of infection success

The fact that the agar-droplet method, which was shown to consistently produce 100% infection in susceptible clones over 9 independent inoculations repeated over time, was able to induce only 56% infection in SCA clones (SCA 6 and SCA 12) and 85% infection in IMC clones (IMC 57 and IMC 67) showed that these clones had high and moderate resistance to

witches' broom disease, respectively, at the first stage of resistance. All the other clones showed near 100% infection in all repetitions, but varied in pathogen growth and colonisation measures. Among the various measures of infection success, the percentage of plants showing swelling had the lowest CV compared to the other measures, which are the percentage of developing brooms and percentage of swellings converted into brooms. The percentage plants showing swelling had a rank correlation of 0.58 with field infection levels recorded by Latchman *et al.* (1999), but this decreased to –0.03, when the extreme values were removed.

Comparison between resistance measures of pathogen growth/ colonisation

Of the measures of pathogen growth and colonisation, incubation period provided the greatest precision at the level of replications (CV = 1.3% for clones and 3.5% for progenies - Figure 2) compared to stem swelling (CV = 11.8 % and 13.5%) and broom weight (CV = 5.4% and 23%). Consequently, the index of differentiation (range of symptoms / $lsd_{0.05}$), which is the ability to discriminate between levels of resistance, was highest for incubation period (38.6 for clones and 35.5 for seedling progenies), followed by broom base diameter (7.14 and 15.3). The index of differentiation decreased, in general, for most measures when the extreme clones or progenies were removed from the analysis, but remained high for incubation period (21 and 34), indicating that this measure is capable of discriminating among both clones and progenies at intermediate levels of resistance.

Heritability measured as the strength of parent-offspring (open-pollinated) regression (Figure 3) was high for both incubation period and broom base diameter (R^2 of linear regression = 86% and 72%, respectively). The data from the clonal trial were re-analysed using single plant observations per clone to determine precision, index of differentiation, and heritability. This provides a simulation of experimental precision and possibility to discriminate when screening segregating progeny, where each genotype is represented by single plants. The precision for incubation period and broom base diameter decreased with single plant estimation, but were acceptable (CV=10% and 13%, respectively) compared to other measures of resistance. Furthermore, the accuracy of means measured by correlation of single plant values to the true mean was high for incubation period (0.93 - 0.96) and broom base diameter (0.75 - 0.95).

When the resistance measures obtained with the agar-droplet inoculation method were correlated to observed field resistance rankings of Latchman *et al.* (1999) using Spearman's rank correlation coefficient, the correlations were high for most measures of pathogen growth, but were particularly high for incubation period (0.95) even when extremely resistant and susceptible clones were removed from the analysis (0.90).

Conclusions

In summary, the agar-droplet method used in this study eliminates escapes. Furthermore, the incubation period as a measure of resistance is simple, precise, accurate and heritable. Firstly, it has been shown to be highly correlated to broom size and moderately correlated to broom frequency, the two epidemiologically important measures. Secondly, it provides the most precise and repeatable measure of resistance and, consequently, best discriminates between levels of resistance to witches' broom disease in cacao. Thirdly, it provides an accurate assessment of resistance even on a single plant basis. Fourthly, this measure shows a strong parent-offspring regression and a strong rank correlation with field resistance.

It is therefore suggested that the agar-droplet inoculation method provides means of assessing resistance at both stages. Percentage infection, measured as the proportion of plants showing swellings, provides the best measure of resistance at stage-I, while incubation period provides the best measure of resistance in stage-II. This will therefore allow selecting for both mechanisms of resistance in breeding programmes. Progeny families can be tested for stage-I resistance using the percentages of plants becoming infected, and individual plants within the better families can be evaluated for stage-II resistance based on incubation period. The results show that incubation period can effectively discriminate between the levels of resistance of individual plants with high precision and accuracy.

SCA clones provide acceptable levels of resistance to pathotype-B of C. *perniciosa*, but not to pathotype-A (Wheeler and Mepsted, 1988). However, Wheeler (1999), investigating the host pathogen interaction between various cacao clones and pathotypes of *C. perniciosa*, did not find sufficient evidence to suggest that interactions exist. If this is true, accumulating genes that retard pathogen growth and colonisation (stage-II) resistance will provide a more durable horizontal resistance to witches' broom pathogens. The fact that incubation period can effectively discriminate between intermediate levels of resistance, even based on single plant estimates, provides hope for accumulation of genes within populations through population enhancement.

The work described above has been accepted for publication in *Plant Pathology and Plant Disease*. The mechanisms of resistance to witches' broom disease and their genetic bases are being further investigated at the Cocoa Research Unit.

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Figure 1: Advanced state of terminal broom development resulting from the application of the agar-droplet inoculation technique

Explanation of acronyms used in this issue

ACRI	American Cocoa Research Institute
BCCCA	Biscuit, Cake, Chocolate and Confectionery Alliance (United Kingdom)
CEPLAC	Commissao Executiva do Plana da Lavoura Cacaueira (Brazil)
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
CRIG	Cocoa Research Institute, Ghana
CRU	Cocoa Research Unit
MCB	Malaysian Cocoa Board
UESC	Universidade Estadual de Santa Cruz
USDA	United States Department of Agriculture

(a) (b)

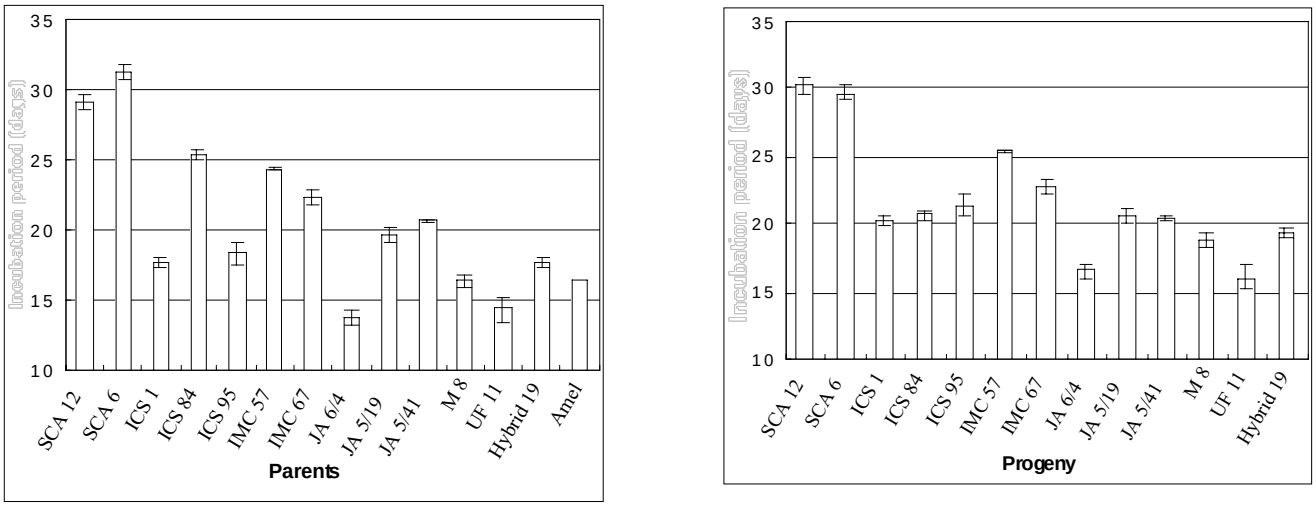

Figure 2: Incubation period (days) in response to inoculation with *C. perniciosa* in (a) 14 clones and (b) 13 open-pollinated progenies of *T. cacao* (bars indicate standard error of means)

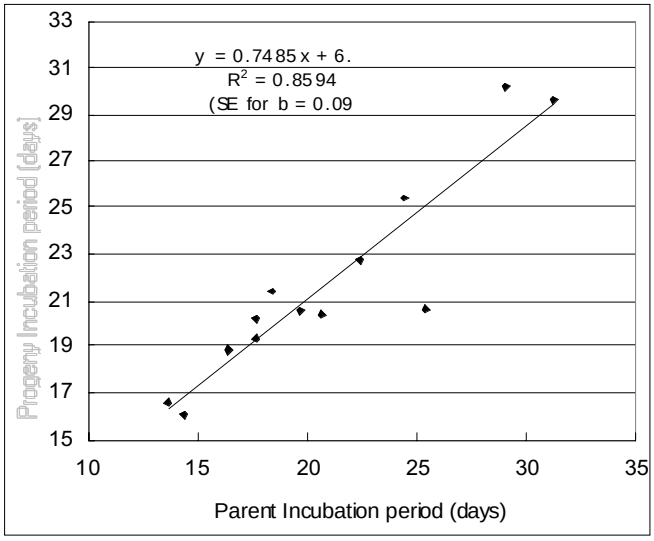

Figure 3: The relationship between parents and their open pollinated progenies of *T. cacao* for incubation period

Opening New Frontiers: Review of a Research Article

entitled

Stable Transformation of *Theobroma cacao* L. and Influence of Matrix Attachment Regions on GFP Expression

In: *Plant Cell Reports* 21: 872-883. (also available from the Guiltinan lab Website: guiltinanlab.cas.psu.edu)

By: Siela Maximova, Carter Miller, Gabriela Antúnez de Mayolo, Sharon Pishak, Ann Young, and Mark J. Guiltinan (2003)

Antonio Figueira

Plant Breeding Laboratory, Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Av. Centenário, 303 - CP 96 - Piracicaba, SP 13400-970, Brazil. e-mail: figueira@cena.usp.br

Cacao had been considered recalcitrant with respect to *in vitro* culture in the past, and progress on breeding application and commercial utilisation of micropropagation have lagged behind other perennial tropical crops, such as coffee, oil palm, and rubber. Despite the early efforts to cultivate cacao *in vitro* (e.g. Archibald, J.F. 1954. *Nature* **173**:351-352), little progress was achieved. It was only in the late 1970s, that the development of somatic embryogenesis from immature zygotic embryos was first reported. But since the explants were zygotic embryos, representing untested genotypes, there was little benefit for clonal propagation. Furthermore, the conversion rates were poor and few plantlets were recovered. Therefore, efforts were directed to test the potential to produce cocoa solids and butter *in vitro*, but with limited success.

Induction of embryogenic cultures from sporophytic (maternal) tissues continued to be investigated, and it was finally described as occurring from nucellus and floral parts in the early 1990s. However, the embryogenic response occurred at a very low frequency and was highly genotype-dependent. Further protocol improvements showed that petals and staminodes were the most responsive explants. A major breakthrough came from the work at the Penn State Cacao Research Laboratory with the development of a two-step induction of somatic embryogenesis from petals and mainly staminodes in two specific media (Li *et al.*, 1998). This new protocol enabled the efficient induction of embryogenic cultures for many genotypes at a reasonable rate. Application of somatic embryogenesis for commercial propagation still requires some fine-tuning to increase the multiplication rate (see Maximova *et al.*, 2002; Traore *et al.*, 2003) and to reduce cost. But it can be used for germplasm preservation *via* cryopreservation of embryogenic genotypes; establishment of seed gardens; to support breeding programmes by multiplying elite individuals; and to establish field trials of sufficient size for agronomic evaluation.

The lack of a reliable regeneration protocol from *in vitro* culture has delayed the development of a transformation system for cacao. Genetic transformation of cacao has been attempted using *Agrobacterium tumefaciens* and particle bombardment approaches, and despite the reports of transient transgene expression, transgenic plants were not regenerated from the transformed cells. Now, another major breakthrough was accomplished by The Penn State Cacao group (Maximova *et al.*, 2003). A method for production of transgenic cacao plants has been developed and proven to be reproducible. Achieving this goal, long the aim of cacao biotechnologists worldwide, opens the door to the use of this powerful technology for research and applied applications in the future.

In this manuscript (Maximova *et al.*, 2003), the Penn State team present the characterisation of an *Agrobacterium tumefaciens* mediated genetic transformation system for cacao, and report the regeneration and reproduction of transgenic cacao plants, a world first. Building on their prior work in the development of a somatic embryogenesis system for cacao, and optimisation of selection conditions to eliminate *Agrobacterium* after transformation (Li *et al.*, 1998; Maximova *et al.*, 2002; Mayolo *et al.*, 2003), the team describes a protocol based on the co-cultivation of secondary somatic embryo cotyledons with *Agrobacterium*. The gene encoding for green fluorescent protein (GFP) was used as a visible marker, and the NPTII gene was also included as a selectable marker. A second DNA construction contains sequences flanking the T-DNA that were isolated from tobacco called Matrix Attachment Regions. Matrix attachment regions are thought to influence transgene expression stability and gene silencing by influencing the local chromatin structure associated with transgene insertion through interactions of proteins in the nuclear matrix. A third construction included a cacao basic chitinase gene under control of a modified CaMV35S promoter. Although the transformation frequency reported is low, ranging from zero to 0.04 transformants per explant, the authors report the reproducible regeneration of transgenic cacao plants. These plants were characterised in some detail; verification of transgene insertion and expression was confirmed.

Beautiful fluorescent images of transgenic cells, embryos and plants are presented. Eighteen independent transgenic lines are reported, and hundreds of clonally reproduced plants were analysed. Interestingly, the conversion of embryos to plantlets was lower for the transgenic embryos then for control non-transformed embryos. The plant heights, stem diameters, leaf numbers and leaf surface areas were carefully monitored and no significant differences between control and transgenic plants were detected. A number of plants from one of the lines were grown to maturity, and pollinated reciprocally, demonstrating full fertility of the plants. A near perfect 1:1 segregation pattern of the GFP gene in the resulting population of 282 progeny indicated that in this line, a single locus of transgene insertion resulted. The successful germination and stable expression of the GFP gene in the next generation of transgenic plants was also reported.

An interesting facet of this paper involves the analysis of the matrix attachment region elements on gene expression and stability. The matrix attachment region elements did not influence the transformation frequency, but did show a significant effect on expression levels of the GFP gene. Quantitative fluorescence microscopy was used to measure GFP expression levels in a large number of embryos. It appears that the matrix attachment region elements contributed to an approximate doubling of GFP gene expression when averaged over the population of lines tested. Furthermore, the variance between different transgenic lines was less for those transformed with the matrix attachment region-containing vector.

The matrix attachment region elements also seem to play a role in suppression of gene silencing. When lines without the matrix attachment region sequences were further propagated via tertiary embryogenesis, in approximately 20% of the cases the resulting embryos exhibited gene silencing, a phenomenon common in transgenic plants thought to be associated with DNA methylation and chromatic remodelling. None of the embryos transformed with the matrix attachment region construction resulted in silenced embryos upon tertiary embryogenesis. Although intriguing, the results with the matrix attachment region elements were obtained on a limited data set, and more data are necessary for a more detailed understanding of their full effects.

Taken together, the manuscript presents an elegant system to transform cacao, indicating the successful transformation and regeneration of transgenic cacao. It also demonstrates the absence of major gross phenotypic changes (including sterility), sometimes associated with the long period of *in vitro* culture of other systems. The article also indicates possible solutions for problems associated with gene silencing. One question not answered in the manuscript is if the chitinase gene included in one of the constructions is expressed and, if so, is there any effect on fungal resistance of the progeny? If this were the case, these plants would provide an excellent molecular tool to study the role of chitinases in plant defense responses.

The authors clearly state that these plants were developed as a research tool only and that they have no intentions of development of transgenic plants for commercial deployment. None of the plants have been transferred outside of the containment greenhouse at Penn State. Certainly, any such developments are long in the future, if ever, and given the current anti-GMO sensitivities of very many consumers, it is possible that transgenic cacao will never see the light of day in a practical sense. However, lack of research on transformation systems for cacao jeopardizes progress in applying this technology to help in basic research on cacao physiology and biochemistry, and to resolve breeding problems. A major example would be the use of cacao genetic transformation to introduce resistance against major diseases and pests, when natural sources of resistance are unknown or limited, such as resistance to Cocoa Swollen Shoot Badnavirus. Another important usage would be the functional analysis of disease resistance candidate genes identified in QTL mapping and genomics programmes.

Other Papers from same Group:
(available from the Guiltinan lab Website: guiltinanlab.cas.psu.edu)

Abdoulaye Traore, Siela N. Maximova and Mark J. Guiltinan (2003). Micropropagation of *Theobroma cacao* L. using somatic embryo-derived plants. *In Vitro Cell and Developmental Biology-Plant*. (in press).

Gabriela Antúnez de Mayolo, Siela N. Maximova, Sharon Pishak and Mark J. Guiltinan (2003). Moxalactam as a counter-selection antibiotic for *Agrobacterium*-mediated transformation and its positive effects on *Theobroma cacao* somatic embryogenesis. *Plant Science* **164**: 607-615.

Li, Z., Abdoulaye Traore, Siela Maximova, and Mark J. Guiltinan (1998). Somatic embryogenesis and plant regeneration from floral explants of cacao (Theobroma cacao L.) using thidiazuron. *In vitro Cell Developmental Biology* **34**:293-299.

Siela N. Maximova, Laurence Alemanno, Ann Young, Abdoulaye Traore, N. Ferrier, and Mark J. Guiltinan (2002). Genotypic variability, efficiency and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L., the chocolate tree. *In Vitro Cell Developmental Biology-Plant* **38**:252-259.

FORTHCOMING EVENT

Fourth INGENIC international Workshop, October 2003

'COCOA BREEDING FOR IMPROVED PRODUCTION SYSTEMS'

Cocoa production is considered to be of low efficiency, with average productivity of about 400 kg per ha. Low yields can be ascribed to disease and pest damage as well as to deficient growing conditions and management. Varieties may be low yielding because of their low yield efficiency (pod yield versus total dry weight production) or because they are poorly adapted to the environment. Cacao varieties may even produce low yields under highly favorable growing conditions, which is an apparent contradiction. Cocoa breeding may have over-emphasised the vegetative vigour of new varieties, which is important for rapid establishment of new plantings, but could be a disadvantage for adult plantings due to strong inter-plant competition.

The objective of the fourth INGENIC Workshop is to analyse the possible contribution of genetic variation within *T. cacao* to improve efficiency of the cacao tree and cocoa production systems in different environments.

The following topics have been selected (not exhaustive):

- How do we develop more efficient cacao trees that are well adapted to their environment?
- Analyses of GxE interactions (sites, planting density, shade conditions, production systems, pruning, soil, rootstocks),
- Analyses of physiological traits related to yield efficiency (light interception, yield/vigour ratio),
- Selection for small trees and compact canopy shape,
- Development of 'dwarfing' rootstocks aiming at smaller and more efficient plants,
- Results on individual tree selection for new clones and on selection of new clones as parents for hybrid varieties,
- The possible role of self-compatibility on pollination efficiency and yield, and
- Any other discussion topics or suggestions on the type of planting materials that are required to improve cacao growing systems.

Interested persons are invited to present papers related to the above topics at the Workshop. These may include research papers, reviews (on specific topics or institutional/national reviews), discussion papers and any proposals or ideas for new activities on these subjects. INGENIC will try to get support for inviting lead speakers who will introduce certain topics or present general reviews on advances made. A tentative programme for the Workshop will be sent around in September 2003.

In addition to the INGENIC workshop, a discussion meeting on cocoa genomics research is planned for the afternoon of Sunday 19 October. This is a follow-up to the discussion meeting co-organised by INGENIC and USDA, held in Miami in January 2002, aiming to further explore possibilities of collaborative activities in this new area of research. The organisers of this discussion meeting will provide further information separately.

INCOPED is also organising a workshop on cocoa pests and diseases, which will coincide with the INGENIC Workshop. INGENIC and INCOPED plan to have a joint opening session and cocktail on the evening of Sunday 19 October.

Information on paper presentations and re-registration for the INGENIC Workshop is provided below.

Papers: Abstracts of papers should be sent to the INGENIC Secretariat before 31 July 2003, and full papers need to be presented at the Workshop (in electronic format and as hard copy).

Date & venue: *Workshop*: 19 to 21 October 2003 in Accra, Ghana (following the 14th. International Cocoa Research Conference).

Cocoa genomics discussion day: 19 October afternoon (same place as the workshop)

Pre-registration: Pre-registration for the workshop is to be done by filling out the information sheet below and sending it back to the INGENIC Secretariat before ***31 August 2003***.

Registration fee: Payment of the registration fee (100 US$), including lunches during the workshop and proceedings, is to be done in cash or with personal cheques at the Workshop registration desk on 19 October.

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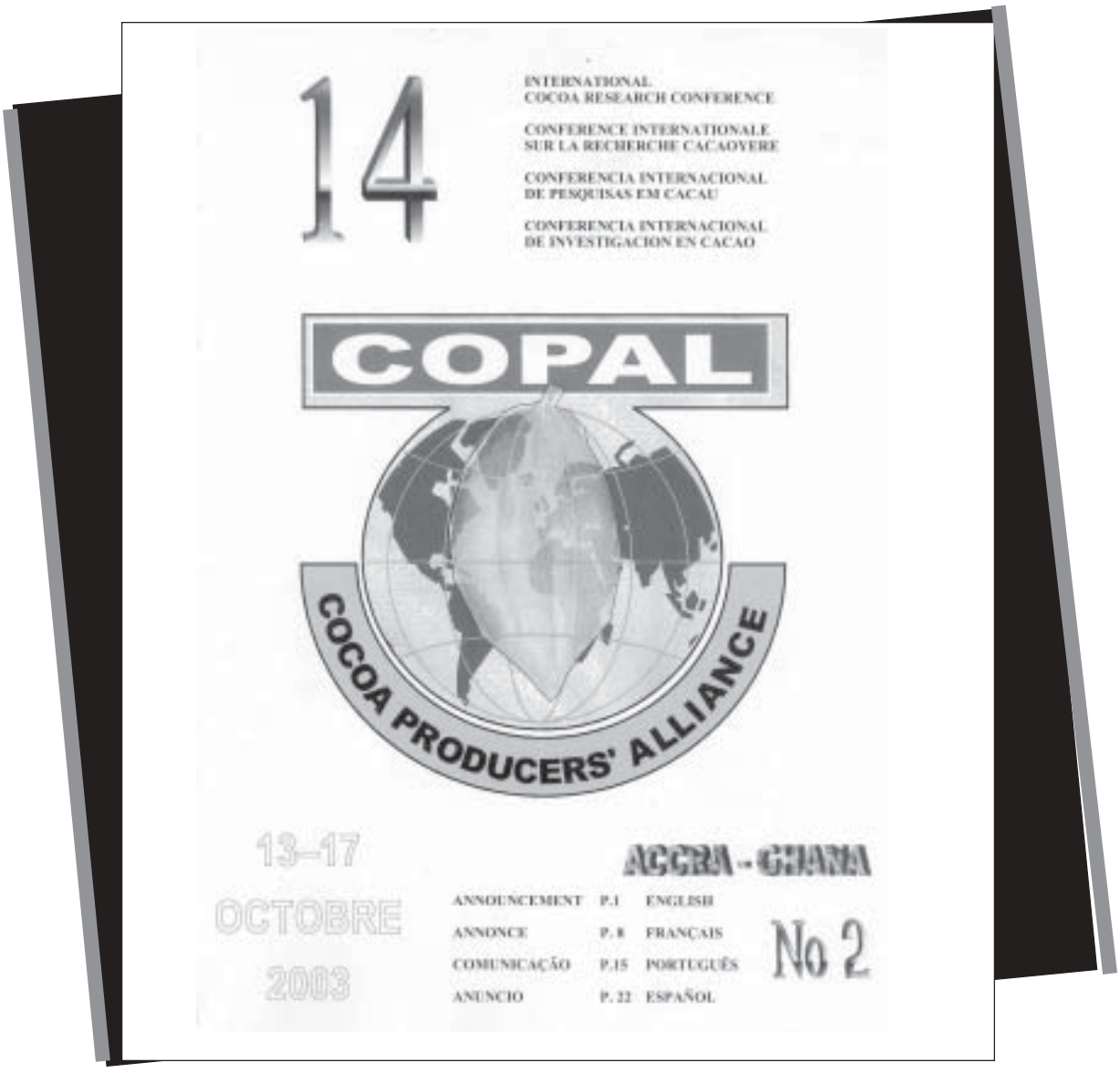


Obituary

Dennis Bruce Murray:
23 July 1915 to 16 March 2003

Victor C. Quesnel

Denis Bruce Murray died on March 16, 2003 and this event should not go unnoticed by his former colleagues, readers of this Newsletter and cocoa research scientists everywhere. He was born on July 23, 1915 of Trinidadian parents in British Guiana. He was educated at Queen's Royal College in Port of Spain, and Christ's College, Cambridge from 1935 to 1938, graduating with a B.A. in Botany. Returning to Trinidad, he studied at the Imperial College of Tropical Agriculture, graduating the following year with the A.I.C.T.A. degree. In 1940, he was posted by the Colonial Office to Nigeria as Botanist in the Department of Agriculture. In 1946, he successfully applied for a transfer back to Trinidad because his wife Betty had been infected with filaria and had been advised to return home. From 1946 to 1950, he was Economic Botanist in the Department of Agriculture in Trinidad and, in 1950, became Senior Plant Physiologist in the Regional Research Centre at I.C.T.A., eventually becoming Head of the Cocoa Research Unit.

I had returned home from studying abroad in 1953, and was Biochemist at the Colonial Microbiological Research Institute (CMRI) when I first met Denis in the late 1950s. Members of staff of the two institutions, ICTA and CMRI, had convened informally to co-ordinate their research programmes on cocoa fermentation. I remember Denis' friendly and accommodating manner as a key factor in the speed with which agreement was reached at a meeting which could easily have become a battle for kudos. When, after closure of CMRI in 1961, I joined the Cocoa Research Unit, I immediately felt "at home". My memory of the event is that Denis was already Head of the Unit then, but I have found no documentation to support this. He was the ideal Head, knowledgeable in his field, decisive, fair in dealings with subordinates, and even-tempered.

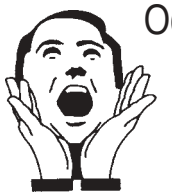
In 1971, when plans were being developed to convert the Unit into an international institute, I and other Research Fellows all thought that Denis would automatically continue as Head of the new organisation. When we learned that this was not so, we all spontaneously protested and stated our views in a letter to the Chairman of the Cocoa Research Advisory Committee. This is a measure of our regard for Denis' capabilities as Head of the Unit. In the end, the plans were not realised, a change of status of the Unit never occurred, and Denis remained as Head until his retirement in October 1975. However, his retirement from the Cocoa Research Unit did not mean a retirement from agriculture, for he promptly accepted the post of Manager at Constance Estate, a large coconut estate in Icacos, south Trinidad, and remained there for about ten years.

Although from 1953 onward Denis' research focused on cacao, his career embraced other important tropical crops. His thesis for the A.I.C.T.A. degree dealt with citrus, his work in Nigeria was with oil palm, and his papers from the Department of Agriculture in Trinidad dealt with rice, citrus and coconuts. Among his notable contributions to cocoa research, specifically in the area of physiology, are his studies on propagation, shade trees, and mineral nutrition and deficiency symptoms with G.K. Maliphant, and root-stock-scion interaction with Prof. F.W. Cope. Denis also collaborated in studies on bean quality. However, Denis is best known for making sense of the interactions of soil moisture, nutrient status and light in their effect on cacao yield. As early as 1956, he was considering the possibility of improving nutrient uptake to the point where cacao could be grown without shade, but with windbreaks so as to obtain maximum yield. As far as I know, the increase in cocoa production in Brasil in the latter half of the 20th century is due in large part to the application in the field of Denis' discoveries in this area of cacao physiology.

Denis married twice. His first wife, Betty Grant, whom he married in 1939, was the mother of his three sons, Graeme, Brian, and Colin. She died in 1959. In 1970, Denis married Marie de Pass, a widow with ten children. To these bereaved relatives I offer my condolences.

INVITATION

You are invited to the
Fourth INGENIC WORKSHOP
on Cocoa Breeding for Improved
Production Systems,
October 19-21, 2003, Accra, Ghana
and the INCOPED Workshop on
Cocoa Pests Diseases,
October 19-21, 2003,
Accra, Ghana



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PRE-REGISTRATION FORM
for the Fourth INGENIC Workshop
'Cocoa Breeding for Improved Production Systems'
19-21 October 2003

Name: _____

Working address: _____

E-mail address: _____

I plan to present a paper, no: ☐ yes: ☐ on the following subject:

_____

_____

_____

_____

I plan to participate also to the discussion meeting on Cocoa Genomics
On 19 October:

yes: ☐ no: ☐