Agronomic Performance and Trueness-to-type of *Coffea arabica* Hybrids Mass-propagated by Somatic Embryogenesis

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SUMMARY

In order to validate a mass propagation process of *Coffea arabica* based on the use of liquid nutrient media, the agronomic performance and trueness-to-type of regenerated trees (selected F1 hybrids) was assessed at a large scale. In a first trial representing 3,000 trees, we determined that the frequency of variants increased exponentially with age of the embryogenic suspension. For the five genotypes, somaclonal variation was low (1.3%) in trees produced from embryogenic callus or 3-month-old cell suspensions and increased in frequency with increasing suspension age (6, 10 and 25% in trees produced from cell suspensions aged 6, 9 and 12 months, respectively). Seven types of phenotypic variants were characterized. Based on vigour and productivity of the regenerated plants, it was possible to class the variants in order of severity of physiological disorders: "Colour of juvenile leaves", "Giant", "Dwarf", "Thick leaf ("Bullata")", "Variegata", "Angustifolia", "Multi-stem". The "Dwarf", "Angustifolia" and "Multi-stem" types were the most frequent among produced plants (1.4, 4.8 and 2.9%, respectively). In a second trial, the agronomic performances of 644 trees derived from somatic embryogenesis were compared with those of normal trees produced from microcuttings for the same four clones. Somaclonal variation has never been observed with microcutting-derived trees. The variant aside (2%), for all clones, the trees had vegetative characteristics, productivity, fertility, and biochemical, mineral and beverage characteristics that were identical to those of the controls. We conclude that propagation of coffee by embryogenic callus or young cell suspensions generated few variants. Moreover, detection of 70% of variants is possible at the nursery stage. Somatic embryogenesis can therefore be considered as a possible mass-propagation technique for coffee, for rapid multiplication of heterozygous structures that should lead to substantial genetic gains compared to traditional pedigree selection schemes.

INTRODUCTION

Coffea arabica L. varieties are traditionally propagated by seed. Given the strong hybrid vigour found when complementary genetic pools (Ethiopian x Catimors) are crossed (Bertrand et al., 1999), substantial agronomic progress is expected from F1 hybrids. Efficient somatic embryogenesis micropropagation procedure would provide a means for the large-scale dissemination of hybrid varieties in clonal form. For instance, 19 clones of *C. arabica* F1 hybrids were multiplied to set up assessment networks in Central America (Etienne et al., 2002). However, the processes need to be optimized before the technique can be applied on an industrial scale. The targets to be reached are (i) a reduction in production costs, (ii) a guarantee that the propagated trees are true-to-type.

The existence of somaclonal variations in trees propagated by somatic embryogenesis has been demonstrated in *Coffea arabica*. Söndahl and Lauritis (1992) estimated that around 10% of trees regenerated from embryogenic callus were variants based on phenotypic

characteristics. Little information is available to date for coffee trees and woody species in general about the impact of culture conditions on the occurrence of somaclonal variations during somatic embryogenesis processes. Various reviews concluded that somaclonal variations are preferentially induced by certain parameters, such as: (1) explant source, its level of ploidy and its number of chromosomes, (2) hormonal factors, i.e. the concentration and type of growth regulators, (3) genotype factors and (4) the age of the culture.

It is well-known that the critical factor is the involvement of a disorganized growth phase, such as the proliferation of an embryogenic cell suspension (Karp, 1991). The aim of our work, using *Coffea arabica* embryogenic suspensions, was to determine how the age of the suspension and the genotype affect somaclonal variation. This article also examines growth and yield performance, bean chemical and biochemical characteristics, and cup quality for four *Coffea arabica* clones produced by this procedure.

MATERIALS AND METHODS

Plant material

Trial 1. Effect of suspension age. The 5 studied clones were derived from five F1 hybrids of *C. arabica* obtained from crosses between the Caturra, Catimor (T8667) and Sarchimor (T5296) cultivated varieties with wild accessions from Ethiopia and Sudan. Clones from the following five crosses: Caturra x Ethiopian N°531, T8667 x Rume Sudan (tree 1), T8667 x Rume Sudan (tree 2), T5296 x Rume Sudan (tree 1), T5296 x Rume Sudan (tree 2) are referred to as clones H1, H2, H3, H4 and H5. Each clone was propagated by embryogenic cell suspension and the plants were regenerated every 3 months, i.e. after 0 (directly from embryogenic callus), 3, 6, 9 and 12 months of proliferation. For field assessments, five blocks were planted, each corresponding to different embryogenic suspension proliferation times. All the informations about this field trial were given previously (Etienne and Bertrand, 2003).

Trial 2. Agronomic performances. The comparative study used clones derived from four F1 hybrid of *Coffea arabica* and referred as clone 1, clone 2, clone 3 and clone 4. Each clone was propagated by both embryogenic cell suspensions and *in vitro* microcuttings. For each of the eight treatments (two micropropagation techniques x four clones), 100 trees were used for agronomic comparisons as previously described (Etienne and Bertrand, 2001).

In vitro multiplication techniques

The embryogenic cell suspension method involved four stages (Etienne and Bertrand, 2003). In Stage 1, immature leaf explants were cultured for 1 month on medium containing 2.26 µM 2,4-D (2,4-dichlorophenoxyacetic acid), 4.92 µM IBA (indole-3-butyric acid) and 9.84 µM iP (iso-pentenyladenine), then transferred for 6 months to a medium containing 4.52 µM 2,4-D and 17.76 µM BAP. An embryogenic callus developped on the explants. In Stage 2, this embryogenic tissue was placed in liquid medium with 4.52 µM 2,4-D and 4.65 µM kinetin. Subsequently, a cell suspension of embryogenic aggregates was produced. Long-term maintenance of the embryogenic suspension culture was achieved by 1-month proliferation cycles, i.e. twelve cycles were completed for the 1-year-old suspensions. In Stage 3, the embryogenic aggregates were transferred for two months to a temporary immersion bioreactor (RITA®, CIRAD, France), containing a regeneration medium supplemented with 17.76 µM BAP. In Stage 4, the regenerated embryos were cultured with a germination medium containing 1.33 µM BAP. Acclimatizable plantlets were obtained after two consecutive 2-month subcultures in the bioreactor with germination medium.

Trueness-to-type of micropropagated trees in the field (Trial 1)

The identification of variants was based on morphological observations after 8 months in the nursery and two years after planting in the field. The plants regenerated *in vitro* that revealed morphological traits that differed from those of the initial clone when acclimatized in the nursery or planted in the field were referred to as 'somaclonal variants'. These somaclonal variants were identified based on height, morphology, leaf shape, productivity, fruit shape, leaf density, stomatal density and guard cell chloroplast number (Krug et al., 1939; Etienne and Bertrand, 2003).

Growth observations, yield and coffee bean characteristics (Trial 2)

The following measurements were taken:

- Stem diameter (5 cm from the ground) after 36 months in the field,
- Increase in stem height between the ninth and thirtieth month in the field,
- Existence of pollen on the stamens of open flowers,
- Number of chloroplastids in the guard cells (determined on 10 leaves per tree for 12 trees taken at random from each treatment (96 trees in total)),
- Production was measured in terms of grams of fresh berries per tree. The data presented were yields for the second year.

The number of fruits per node was estimated from the eight most heavily bearing nodes.

Frequency of peaberries in a sub-sample of 100 green fruits collected 6 months after flowering from the eight most heavily bearing nodes.

Frequency of floating mature berries in a sub-sample of 200 ripe fruits after immersion in water.

Bean weight of the coffee produced by each tree was estimated by measuring the dry weight of 200 beans from mature berries.

Bean chemical analyses

For 12 trees from each treatment (96 trees in total), 250 g samples of green coffee were collected after screening through a size 17 sieve and eliminating most defective beans. The following traits were measured for each tree separately: Sucrose, chlorogenic acids, caffeine, trigonelline, and fat contents of beans were obtained through chemical analysis after grinding the beans following the method of Guyot et al. (1988). Each chemical analysis was achieved by near infrared spectrometry reflectance on green coffee after grinding (NIR spectrometer system (model 6500, NIRSystem, Inc. 1201 Tech Road Silver Spring, MD 20904) driven by NIRS2 (4.0) software (Intrasoft International LLC, RD 109, Sellers Lane, Port Matilda, PA 16870).

Bean mineral contents

Chemical analysis of potassium, magnesium, calcium, zinc and copper were carried out by atomic absorption spectrophotometry (AAnalysis 100, Perkin Elmer) after wet digestion with a mixture of nitric and perchloric acid (5:1). The aluminium content was measured by the same method after dry digestion. The phosphorus content was determined by a colorimetric method developing molibdene blue by UV/V spectrophotometry at 660 nm (LAMBDA1,

Perkin Elmer). Total nitrogen was determined by the Kjeldahl method (Jones and Case, 1990).

Organoleptic analysis

Samples were prepared from very ripe, healthy berries harvested from the upper branches of 12 trees during the harvesting peak. The 2 kg coffee samples were prepared by the wet method (pulping, fermentation and drying). One-kilogram samples of green coffee were collected after screening through a size 17 sieve and eliminating most defective beans. After roasting for 6-7 mn, cup quality tests were carried out on an infusion prepared using 12 g of roasted coffee (Van der vossen, 1985). A panel of eight persons tasted 120ml of infusion. The main taste and flavour attributes (aroma, body, acidity) were estimated using scales ranging from 0 to 5 where 0=nill, 1 = very light, 2 = light, 3= frank, 4= strong and 5 = very strong. There was also an overall standard for cup quality ranging from 0 to 5 where 0= not good for drinking, 1 = bad, 2 = regular, 3 = good, 4 = very good, 5 = excellent.

RESULTS

Types of variants found (Trial 1)

Seven types of variants were found and described (Table 1, Figure 1). Apart from the "Multi-stem" variant (Figure 1H), all the other types of variants corresponded to the descriptions of mutations seen in seed progenies (Cramer, 1913; Krug et al., 1939). Histological markers of the "Dwarf" and "Thick leaf" variants like stomatal density or chloroplast number per guard cell were found. We showed that these two phenotypes and the "Angustifolia" phenotype (Figure 1E) can also be easily characterized by the leaf shape (Table 1). Most variations caused a substantial drop in tree vigour and productivity. However, two phenotypes, one involving a change in juvenile leaf colour (Figure 1B), the other producing a "Giant" phenotype (Figure 1D) had normal vigour as well as productivity.

The "Variegata", "Dwarf", "Angustifolia" and "Multi-stem" variants were generally less vigorous than normal plants. On the contrary, the "Giant" and "Thick leaf" variants were remarkable by a taller height. Apical dominance was weak in the "Multi-stem" variant, which was characterized by abnormally high branching as a result of the production of 2 to 4 stems from cauline buds (Figure 1H). This phenotype was expressed from juvenile stages, making it easy to detect in the nursery. The plants died in the field in strong sunlight.

For "Variegata", "Giant", "Bullata" and "Colour of juvenile leaves", the frequency did not exceed 0.3%. The "Dwarf" variant, which is a variant affecting the size and productivity of the tree, exceeded 1%. The "Multi-stem" and "Angustifolia" variants amounted to 2 and 4%, respectively.

Frequency of variants depending on suspension age (Trial 1)

There was no difference between 0 and 3 months of suspension culture in the proportion of regenerated variants produced (Figure 2). However, from 6 months of suspension onward, the average proportion of variants calculated for all clones significantly increased exponentially with suspension age (Figure 2), and can be modelled as: Frequency = $0.99\ e^{0.267t}$, $r^2 = 0.99$ (where t = suspension age). Nevertheless, the use of this model masks the considerable disparities existing between genotypes.

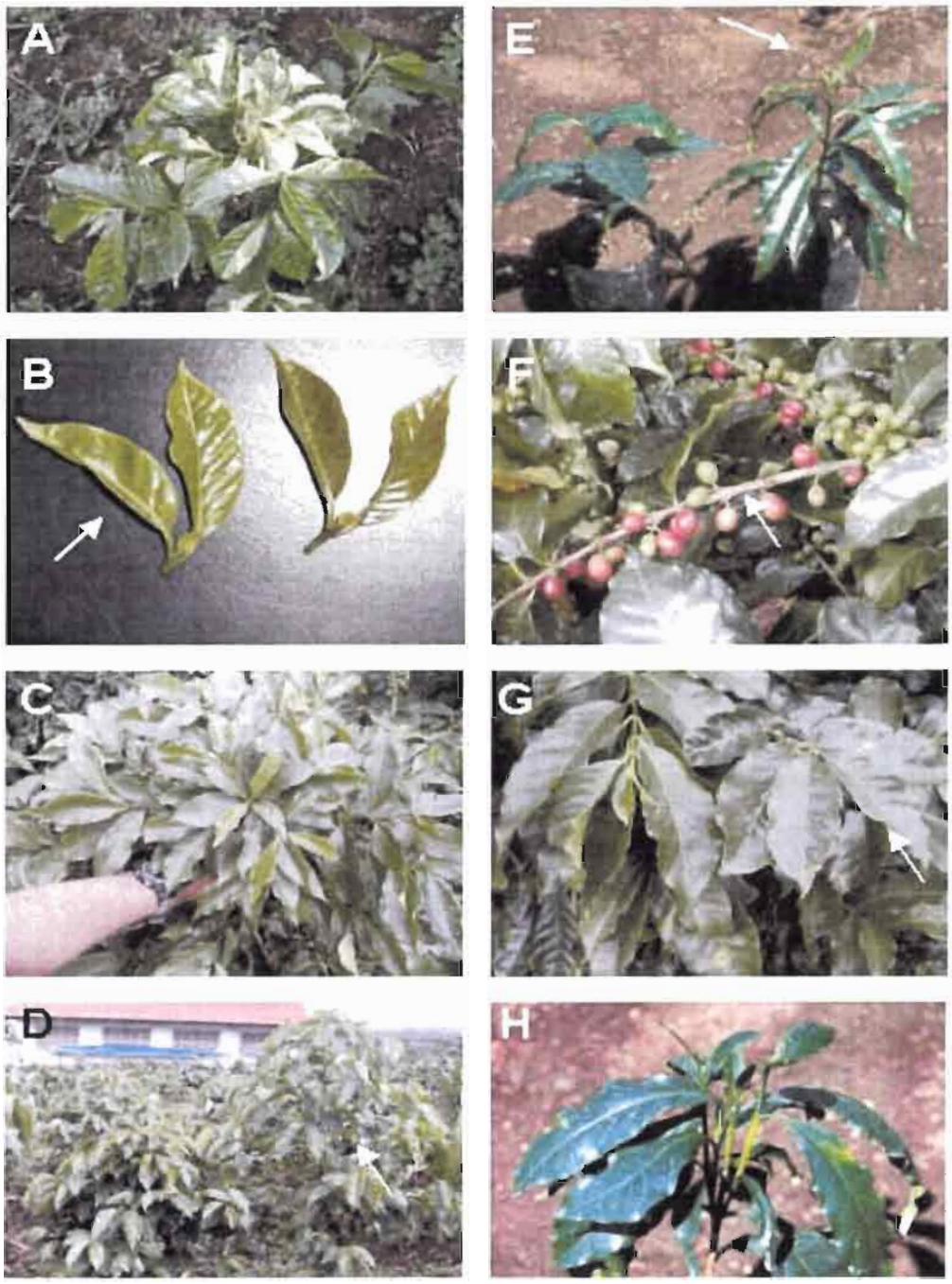

Figure 1. Aspect of the seven phenotypic variations observed in *Coffea arabica* among plants derived from embryogenic cell suspension. The arrows indicate the variant plant material. A) 'Variegata' variant. B) Somaclonal variation for the colour of juvenile leaves (bronze normal leaves on the right and green variant leaves on the left). C) 'Dwarf' variant characterized by a compact phenotype and small leaves. D) 'Giant' variant in the field. E) 'Angustifolia' variant (on the right) with elongated leaves. F) The arrow indicates a branch of the 'Thick-leaf' ('Bullata') variant bearing few fruits of large size. In the background, aspect of a branch from a normal plant. G) On the right, the arrow shows the rounded, lustreless and thick leaves of the 'Thick leaf' variant. On the left, aspect of normal leaves of the same hybrid. H) 'Multi-stem' variant in nursery in which can be observed the emergence of four stems from the cauline bud.

Table 1. Description of seven *Coffea arabica* variant phenotypes based on morphological criteria and productivity. Means followed by the same suffix are not significantly different at $P \leq 0.05$.

Variant	Phenotype	Measurement	Value for variants	Value for controls
Variegata	Variegated leaves, decreased tree vigor	Productivity (g plant^{-1})	484.5 b	1298 a
Colour of juvenile leaves	Developing leaves changed from green to bronze in color, tree vigor unaffected	Productivity (g plant^{-1})	1054 a	1098 a
Dwarf	Small leaves, small trees	Leaf length (cm) Leaf width (cm) Productivity (g plant^{-1})	10.40 b 4.45 b 825 b	13.57 a 5.89 a 1179 a
Giant	Normal leaves, taller trees	Tree height (cm) Productivity (g plant^{-1})	260 a 1268 a	195 b 1324 a
Angustifolia	Elongated leaves, fewer or no domatia, longer internodes, taller trees, decrease of tree vigor	Leaf width (cm) Domatias (nb/leaf) Tree height (cm) Productivity (g plant^{-1})	6.50 b 1.72 b 253 a 323 b	7.23 a 10.4 a 190 b 1245 a
Thick leaf (Bullata)	Rounded, lustreless and thick leaves, starry flowers, large-sized fruits	Leaf length (cm) Leaf width (cm) Leaf width/ leaf length Productivity (g plant^{-1})	13.21 b 7.85 a 1.69 b 678 b	14.37 a 6.70 b 2.15 a 1198 a
Multi-stems	Highly branched, died in the field	Nb of stems emerging from the cauline bud Productivity (g plant^{-1})	2 to 4 0 b	1 1590 a

Number of variants observed depending on the genotypes (Trial 1)

The genotypes H4 and H5, which had over 18% of variants (evaluation including plants from all cell suspension ages), were statistically different from genotypes H1 and H3, which had around 5% of variants. Genotype H2, with about 9% of variants, fell between these two groups. However, the differences between genotypes only appeared from 6 months of suspension onward (Figure 2). From 6 months onward, somaclonal variations were then 10% for genotypes H2 and H3, whereas the proportion of variants for genotypes H1, H4 and H5 was similar to that seen at 0 and 3 months. For the 9-month suspension, the proportions of variants ranged from 6.25% for H5 to 14.63% for H2, but the differences between genotypes, were not significant (χ^2_{obs} = 5.42 and $\chi^2_{0.95}$ = 9.49); for the 12-month suspension, the differences between genotypes were significant. For genotypes H4 and H5, the proportions of variants were 95 and 83% respectively. These two hybrids formed a group significantly different ($P < 0.0001$) from the group of genotypes H1, H2 and H3 (proportions of variants of 8.53, 18 and 5.91%, respectively).

Agronomic characteristics (Trial 2)

For the eight studied variables, representing vegetative, production and fertility characteristics, the analysis of variance indicated that there was no significant differences between trees produced from microcuttings and those produced by somatic embryogenesis (Etienne and Bertrand, 2001) (Figures 3A, B). No clone x micropropagation technique interaction was found for 7 of the variables. There was a significant interaction for the percentage of peaberries but it seems to have little meaning since the percentage of peaberries

was higher in the microcuttings than in the somatic embryos for clone 3. Flowering was uniform irrespective of clone and micropropation technique. The observation of pollen on the stamens did not reveal any sterile males. The data also indicated the existence of a strong "clone effect" for all the variables considered.

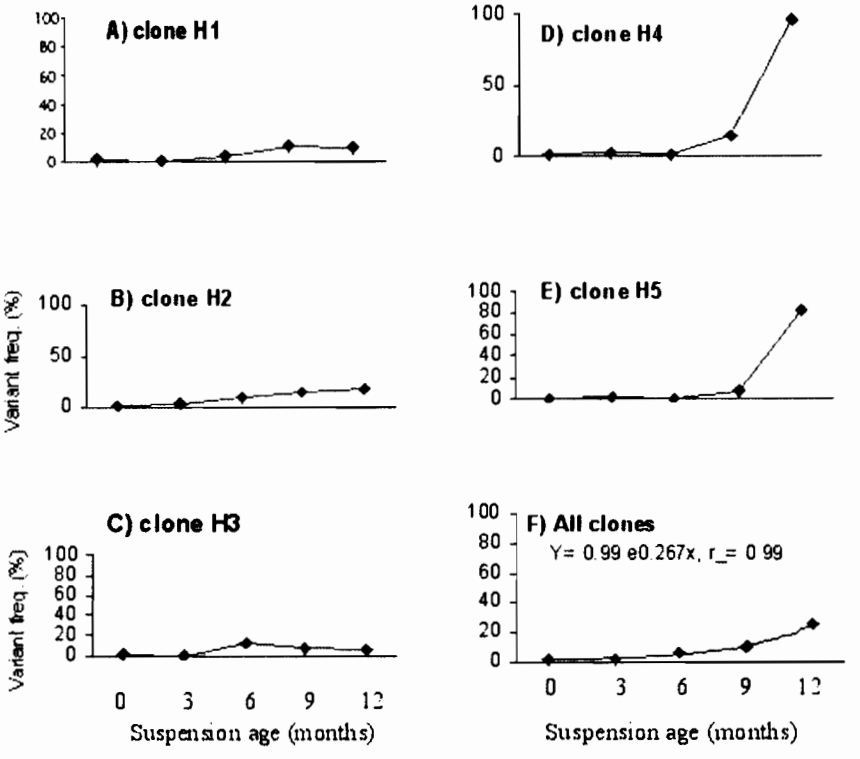

Figure 2. A, B, C, D, E) Evolution of somaclonal variant frequencies depending on suspension age for five genotypes (clones H1 to H5); F) evolution of the average variant frequency obtained with all clones.

Seed biochemical and mineral characteristics, and cup quality (Trial 2)

For the 17 variables studied, representing the biochemical and mineral characteristics of the seeds, along with the organoleptic characteristics of the infusion (Etienne and Bertrand, 2001) (Figures 3C,D), no significant difference was found between plants obtained from microcuttings or from embryogenic cell suspensions. There was therefore no "technique" effect. Differences were observed in a few cases, for biochemical characteristics only, for the same clone and for both techniques. The differences involved caffeine content for clone 1, chlorogenic acid content for clone 2 and sucrose content for clone 2. These slight differences seemed to be down to chance and did not always go in the same direction. They could not be explained by the propagation technique used. Moreover, no interaction was found between the micropropagation technique and the clone used. The analysis of variance for these results also revealed a strong clone effect.

DISCUSSION

This work confirms the existence of somaclonal variations in trees of *Coffea arabica* propagated by somatic embryogenesis, as already reported (Söndahl and Lauritis, 1992). However, the frequency of "off-types" (2.1%) was below the 10% reported by those authors.

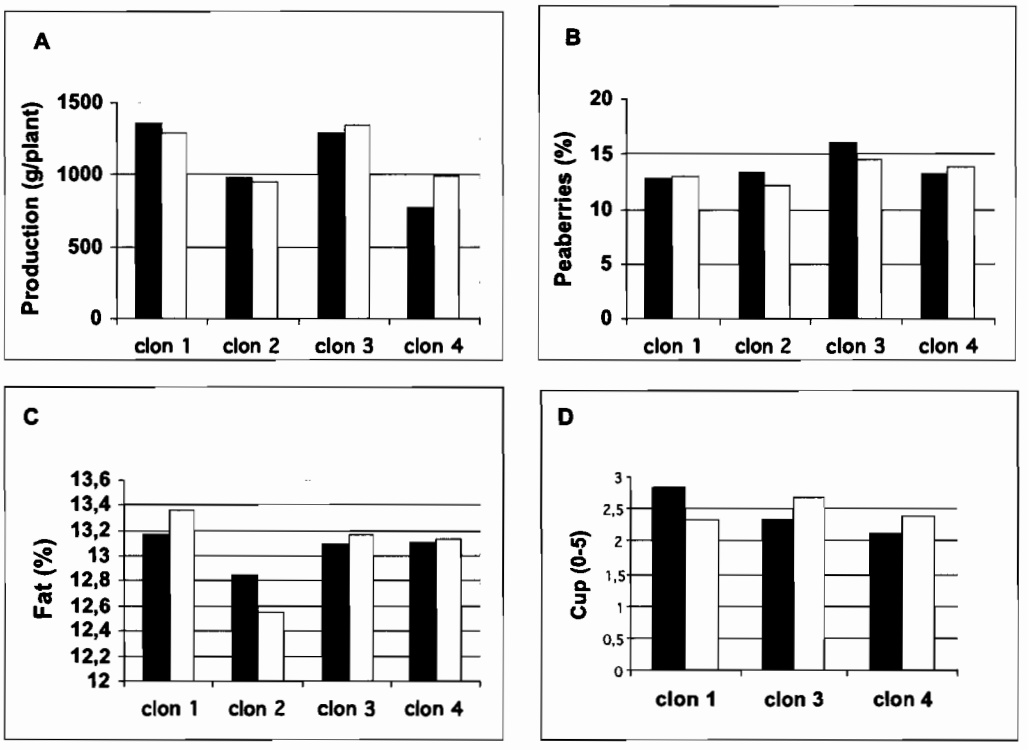

Figure 3. Comparison of some agronomic characteristics (production, percentage of peaberries, bean fat content and beverage quality) for four Coffea arabica clones micropropagated by in vitro microcuttings and embryogenic cell suspensions. Complete data were given previously (Etienne and Bertrand, 2001).

Factors affecting the variant rate

It has frequently been shown that the proportion of somaclonal variants increases with the number of multiplication cycles, or with the length of culture time. This has also been shown for some somatic embryogenesis procedures (Morrish et al., 1983; Symillides et al., 1995; Henry et al., 1996). The existence of somaclonal variants in the progeny of coffee plants propagated by somatic embryogenesis has been documented (Söndahl and Lauritis, 1992; Etienne and Bertrand, 2001; Etienne and Bertrand, 2003). The role of genotype was demonstrated in coffee, but the roles of other culture parameters such as growth regulators and in particular, culture age, were not established. We have shown that the age of *Coffea arabica* embryogenic suspensions affects variant rate. For all genotypes studied, the frequency of variants increased exponentialy. For true-to-type multiplication, it will be essential to restrict embryogenic material multiplication times to less than 6 months. However, the initial stages of cell dedifferentiation and embryogenic callus induction (stage 1) also proved to be mutagenic, as some variants were regenerated from callus of most of the genotypes. Similar findings have been reported for coffee (Söndahl and Bragin, 1991), tomato (Ramulu, 1991) and banana, where it was found to be associated with excessive use of auxin analogs (Shchukin et al., 1997). As was demonstrated in barley cultures (Ziauddin and Kasha, 1990), it is possible that culture age is associated with prolonged exposure to 2,4-D (1 mg/l).

All genotypes in the study exhibited somaclonal variation, however, there were differences between genotypes, primarily in the intensity of the phenomenon. The existence of a genotype effect on somaclonal variation in *C. arabica* has already been shown in F1 hybrids (Etienne and Bertrand, 2001) and in nine widely cultivated varieties (Söndahl and Bragin, 1991). Söndahl and Bragin (1991) showed that plants propagated by somatic embryogenesis, without

an embryogenic suspension proliferation stage, all had variants but with high and variable frequencies ranging from 3 to 39%. Hybrids belonging to the same family, such as H2 and H3 (cross T8667 x Rume Sudan), as well as H4 and H5 (cross T5296 x Rume Sudan) performed in a similar way with respect to frequency of variants, the timing of their appearance, and the type of variants observed (Etienne and Bertrand, 2003). These observations support the hypothesis of a marked influence of genotype.

Agronomic characteristics

Apart from examining the trueness-to-type of plants, it is important to test plants obtained by somatic embryogenesis in conventional agronomic trials. Indeed, somaclonal variants sometimes also reveal themselves through more or less intense flowering, or lower yields. For instance, in wheat, lines obtained from somatic embryos produced 11% less on average than the breeder lines, and had 3.8% fewer spikelets per spike, 6.5% fewer kernels per spike (Hanson et al., 1994). Likewise, in tall fescue (*Festuca arundinacea* Schreb.), there are no easily detectable differences for identifying somaclonal variation, but total biomass and seed yields differ between seedling plants and those obtained by somatic embryogenesis (Roylance et al., 1994). Our work on *Coffea arabica* hybrids shows that, if the 2.1% of variants that were easily detectable in the field through their morphology, which differed considerably from normal, were not taken into account, no difference was found in the main agronomic characteristics between trees produced from embryogenic suspensions and trees obtained from microcuttings with the criterions studied. The trees revealed vegetative characteristics, productivity, fertility, bean biochemical, mineral and organoleptic characteristics that were identical to those of the controls. Similar results were obtained with banana (Côte et al., 2000) and barley (Baillie et al., 1992).

All the results obtained show that propagation of coffee trees from young embryogenic cell suspensions generates few variants and that, apart from those variants, the agronomic performance of trees propagated by that technique is identical to that of trees obtained from microcuttings. Somatic embryogenesis can therefore be envisaged as a new propagation technique for coffee, for rapid and mass dissemination of heterozygous structures that should lead to substantial genetic gains compared to traditional pedigree selection schemes.

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