

**Development of SSR markers and identification of
markers linked to root and associated physiological
traits in *Coffea canephora* Pierre ex. Froehner**

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PAK 2030**

**DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE
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traits in *Coffea canephora* Pierre ex. Froehner**

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PAK 2030**

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award of the Degree of**

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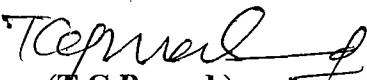
*Dedicated to my
Parents
and
my Mentor*

**DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES, BANGALORE**

CERTIFICATE

This is to certify that the thesis entitled “**Development of SSR markers and identification of markers linked to root and associated physiological traits in *coffea canephora* Pierre ex. Froehner**” submitted in partial fulfillment of Doctor of Philosophy in Crop Physiology to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by **Mr. DEVARAJA ACHAR, A.M** bearing I.D No. **PAK 2030** under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

Bangalore
5th July 2007


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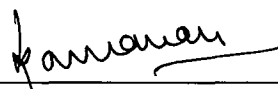
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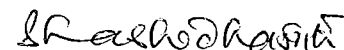
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“Development of SSR markers and identification of markers linked to root and associated physiological traits in *Coffea canephora* Pierre ex. Froehner”

Abstract

Moisture stress limits potential productivity in crops, including Coffee. It is emphasized that introgression of relevant traits such as root traits and water use efficiency is essential to achieve an overall improvement in productivity under water limited conditions. Being quantitatively inherited, marker associated selection approach would be most suitable. Stable isotopes have also been standardized as high throughput screening options. In an earlier study, a mapping population (L1 valley x S.3334) of 134 lines was developed and phenotyped for root traits, $\Delta^{13}\text{C}$ and other parameters. Variability in WUE was also recorded by gravimetry to document significant variability normally distributed around mean.

Continuing this study in the present investigation, an attempt was made to generate microsatellite markers through SSR enrichment strategy. A total of 150 clones were sequenced and 20 of them were found to have locus specific amplification with two markers exhibiting polymorphism between S.3334 and L1 valley. Additionally, 32 SSRs from public domain were screened and seven were found polymorphic. Screening of mapping population with SSR and 41 RAPDs yielded 79 polymorphic loci. Marker trait association was performed by Single marker analysis. Two RAPD markers that associated with root length (OPS₁₈₅₀) and root dry weight (OPL₁₂₀₀) were converted into SCAR markers.

To validate these markers a separate mapping population segregating for root traits was developed by crossing DR-14 (low) and Madagascar (high) which were identified after screening 35 accessions for root traits and WUE in two separate gravimetric experiments. The mapping population consisting of 127 individuals was phenotyped gravimetrically to record significant variability. Selective genotyping of 10 low and high root length & root dry weight types was carried out using SCAR markers. Although the markers revealed an association, a tight association was not evident. Further experiments are underway with more markers.


Signature of the major advisor

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ABBREVIATIONS

AFLP	- Amplified Fragment Length Polymorphism
CID	- Carbon Isotope Discrimination
LA	- Leaf Area
LAD	- Leaf Area Duration
LAI	- Leaf Area Index
MTR	- Mean Transpiration Rate
NAR	- Net Assimilation Rate
RAPD	- Random Amplified Polymorphic DNA
RFLP	- Random Fragment Length Polymorphism
RH	- Relative Humidity
RWC	- Relative Water Content
SCARs	- Sequence Characterized Amplified Regions
SLA	- Specific Leaf Area
SSR	- Simple Sequence Repeats
STSs	- Sequence Tagged Sites
T	- Transpiration rate
TDM	- Total Dry Matter
TER	- Transpiration Ratio
WUE	- Water Use Efficiency
$\Delta^{13}\text{C}$	- Discrimination against ^{13}C

EDTA	: Ethylene Diamine Tetra Acetic acid
TE	: Tris EDTA buffer (10 mM Tris-Hcl and 1 mM EDTA, pH 8.0)
PVP	: Poly Vinyl Pyrolidone
CTAB	: Cetyl Trimethyl Ammonium Bromide
dNTP	: dinitro-triphosphate
M	: Molar
μM	: micromolar
mM	: millimolar
g	: gram
mg	: milligram
μg	: micro gram
ng	: nanogram
pg	: picogram
ml	: milliliter
μl	: microliter
pmol	: picomoles
rpm	: rotation per minute
bp	: base pairs
kb	: kilo base pairs
Taq	<i>Thermus aquaticus</i>

Introduction

I. INTRODUCTION

Coffee is one of the important beverage crops and also second important commodity in the global trade. It is grown in over 80 countries in the tropical and subtropical regions of the world contributing substantially to their national economies. Approximately 100 species of the genus *Coffea* L. (Rubiaceae) are recognized. The most commonly important of these are *C. arabica* L., also known as arabica coffee, and *C. canephora* (Pierre ex Froehner), known as robusta coffee. *C. arabica* is a tetraploid ($2n=4x=44$) and self-fertile species in the *Coffea* genus. All other *Coffea* species, including *C. canephora* are diploid ($2n = 22$) and generally self incompatible (Charrier & Berthaud, 1985).

Coffea canephora (Robusta Coffee; syn. *Coffea robusta*) is a cultivated species of coffee, which has its origins in western Africa. It is grown mostly in Africa and Brazil, where it is often called Conillon. It is also grown in Southeast Asia where French colonists introduced the crop to Vietnam in the late 19th Century and now has exports second to Brazil. The Canephora plant has a greater crop yield than that of Arabica and is less susceptible to pest and disease, but is said to have a taste much bitter than Arabica. The term 'robusta' is actually the name of a widely grown variety of this species. It is a robust shrub or small tree growing up to 10 meters in height, but with a shallow root system. *C. canephora* is diploid ($2n=22$) and self-sterile, producing many different forms and varieties in the wild.

The gene sequences in *C. arabica* and *C. canephora* are about 50% different from each other. The *C. canephora* is much more resistant to disease and insect damage than the *C. arabica* species. The difference in genes could be the key candidates to develop hardier arabica varieties. It is indigenous to African regions characterized by abundantly distributed rainfall and atmospheric humidity frequently approaching saturation (Willson, 1999). For this reason, robusta coffee probably evolved as a water spender species (DaMatta and Rena, 2001).

India is the second largest coffee producer in the Asia-Pacific region and responsible for 25% of Asia's coffee production. The majority of India's coffee production takes place in the states of Karnataka (68.6%), Kerala (23.6%), and Tamilnadu (6.6%).

Most of the coffee tracts of South India are under influence of South West and North East Monsoons. The South West monsoon are more predominant in Kerala and Karnataka, where as North East Monsoon is at Tamilnadu. However mid November to April, there is a dry period in the areas of South west monsoon. Further, the high temperature coupled with poor subsoil moisture during summer season substantially reduces the yield which have been considered as major limiting factors. Drought and unfavourable temperatures are the major climatic limitations for coffee production. These limitations are expected to become increasingly important in several coffee growing regions due to the recognized changes in global climate, and also because coffee cultivation has spread towards marginal lands.

The physiology of plant responses to drought stress is rather complex, showing different modifications following soil drying. The dynamics of soil water depletion, changes in water demand from the atmosphere, as well as plant growth and the phenological state in which water deficit is developed, are sources of the wide variation in plant responses to drought (Medrano et al., 1998). Furthermore, particularly in the tropics, drought episodes are remarkably aggravated by both high solar radiation and temperature, so drought should be accounted for as a multidimensional stress (DaMatta, 2003).

There is a need of great attention for separating the productivity under drought, which is important for agricultural plants, from survival mechanisms (Richards, 1997). One approach to improve crop performance in water-limited environments is to select for genotypes that have improved yield in these environments (Mullet and Whitsitt 1997). A complementary approach to improve plant performance for drought-prone regions involves the identification and selection of traits that contribute to drought tolerance or water use efficiency (WUE). A partial list of potentially important traits might include water-extraction efficiency, WUE, hydraulic conductance, osmotic and elastic adjustments, and modulation of leaf area. Most of these traits are complex and their control and molecular basis are very important in trait introgression.

A major component of differential adaptation to drought among arabica and robusta coffee genotypes appears to be behavioural, and may be governed by rates of water use or efficiency of extraction of soil water (Meinzer *et al.*, 1990b; DaMatta *et al.*, 2003; Pinheiro, 2004). Therefore, physiological and morphological characteristics, such as, stomatal conductance (g_s), crown architecture, root depth and WUE, should be recommended as potential traits for selecting coffee genotypes with superior performance under drought conditions.

Rooting capacity is one of the important drought avoidance traits. Large genotypic differences in growth and productivity in response to water deficit in many clones of robusta coffee are found to be associated with differences in efficiency of root traits and WUE (Piheiro *et al.*, 2005). Nevertheless, robusta coffee evolved as a water splendor species (DaMatta and Rena, 2001) and it has been largely cultivated in regions where water availability constitutes the major environmental constraint affecting crop production. Therefore selection for a well-developed root system with long, thick roots should improve the drought tolerance, because the plant would avoid water stress by absorbing water stored in the deep soil layers (Yoshida and Hasegawa, 1982). In fact, in perennial plant species the significance of roots has been shown in grafting studies (White and Castillo, 1989). Several reports demonstrate the existence of variation in the root traits such as the root volume and the root length spread density contributes to plants total performance under drought conditions (Edmeades *et al.*, 1999).

In any case accumulation of compatible solutes under water deficit conditions appears to be a sensitive indicator of leaf water status (Maestri *et al.*, 1995). Therefore osmotic adjustment is not a general trait observed in coffee under drought condition. However the high relative water content (RWC) maintained by coffee leaves under dehydrating conditions (Nunes, 1976; Josis *et al.*, 1983; DaMatta *et al.*, 1993) appears to be a means of drought avoiding rather than tolerating and hence conservation of high symplastic volume is directly related to maintenance of gas exchange than turgor (Meinnzer *et al.*, 1990b). Root characteristics and growth play a crucial role in maintaining the water supply to the plant, and drought adapted plants are often characterized by deep and

vigorous root system. This indicates rooting capacity is directed to improve resistance in coffee. Understanding such mechanisms in coffee genotypes, naturally adapted to drought have been shown to potentially contribute to yield under drought conditions (DaMatta, 2004b).

Despite ample genetic variability for various parameters of root architecture, its genetic improvement using conventional selection based on phenotype in breeding programmes is unfeasible (O'Toole, 1989). Screening genotypes for root traits has rarely been incorporated into breeding programmes, due to the time consuming nature of the work and complex inheritance pattern (quantitative genetic control). Root characteristics are cumbersome to evaluate when compared to shoot or above ground characters and sometimes result in their damage while phenotyping leading to erroneous values. However DNA based molecular markers revealed this problem by amplifying specific trait controlled region. O'Toole (1989) opined that molecular marker technology shows 'near term use for improving drought resistance breeding. Hence molecular markers can be used to identify quantitative trait loci (QTL) for rooting ability and these can be selected more easily in a breeding programme than the traits themselves (Wang and Paterson, 1994).

QTL mapping provides a powerful tool for conducting physiological and genetic research to understand and possibly improve drought resistance. It eases screening for traits that are difficult to quantify and are influenced by environmental 'stimuli' (Hanson et al., 1990). Now it is realized that one of the options to progress in this area is to develop tightly linked markers to QTLs and subsequently the candidate genes controlling traits of agronomic importance. Such an approach is relevant for multigene regulated traits such as rooting pattern and WUE in perennial plant species. However molecular markers like AFLP, SSR, SCAR, CAPS, STS and other markers provide fairly comprehensive genomic coverage, have locus identity, and they are multi-allelic. Indeed these markers could be used in the trait validation.

In view of the relevance of such novel approaches, present investigation was taken to identify and validate molecular markers linked to root & other associated physiological traits in *Coffea canephora* mapping population. The major objectives of the investigation are as follows.

1. Development of SSR markers and their characterization in coffee species.
2. Molecular analysis of *Coffea canephora* mapping population by RAPD and SSR markers.
3. Development of locus specific sequence characterized amplified region (SCAR) marker from RAPD markers linked to root trait.
4. Validation of SCAR markers in the other mapping population of *Coffea canephora*.

Review of literature

II. REVIEW OF LITERATURE

Coffee is one of the most valuable agricultural commodities and ranks second on international trade exchanges. The genus *Coffea* belongs to the Rubiaceae family, which includes other important plants. About 100 species of the genus *Coffea* have been identified so far (Bridson and Verdcourt, 1988); most of them are trees and shrubs growing at low altitudes in the tropical rain forests of Africa and Asia. Commercially, production relies only on two species, *Coffea arabica* L and *Coffea canephora* Pierre ex Froehner,

Two species are mainly cultivated all over the tropical world: Arabica is a self fertile, produced from *Coffea arabica* ($2n = 4x = 44$), which grows in highlands, and Robusta is self sterile, produced from *Coffea canephora* ($2n = 22$), which grows in low lands, however the arabic and robusta coffee respectively contributing 66 & 34% of the global coffee production (DaMatta. 2006)

Robusta coffee is native to low land forest of the Congo River basin (Africa) with a typical equatorial climate in which average temperature is between 24 and 26⁰C. It is considered as an important source of disease resistance genes and has been transferred into *C. arabica* gene pool via natural and inter-specific hybridization between the two species (Coste 1992 and Willson 1999).

However the Robusta coffee cultivation is increasingly expanded towards marginal and degraded lands where water availability constitutes one of the major problems. In these areas, during the dry season, evaporation will far exceed than rainfall and within the rainy season, rainfall pattern & amount are unpredictable to a greater extent. Such conditions could be largely expected to restrict the potential productivity of Robusta coffee (DaMatta *et al* 2003). In fact, local experience has shown that the crop yield under rainfed conditions may decrease by about 40% or even as much as by 80% in dry years. Under such situation the productivity level could be recovered by employing irrigation schedules. Furthermore, plant response to drought stress is rather more complex, in fact follows the either of the drought resistance mechanisms, namely drought escape, drought avoidance & drought tolerance (Kramer and Boyer. 1995).

The strongest limitations of the coffee production are frost and drought. Frost easily damages and eventually completely kills the coffee tree as indeed occurs from time to time either in southern Brazil or closer to the equator, at altitudes around 2000 m and above. However drought episodes are much more frequent than frosts, so drought is considered the major environmental stress affecting coffee production in most coffee growing countries. Particularly in the tropics, drought episodes are remarkably aggravated by both high solar radiation and temperature, so drought should be accounted for as a multidimensional stress (DaMatta, 2003). As discussed by Mullet and Whitsitt (1997), one approach to improve crop performance in water-limited environments is to select for genotypes that have improved yield in these environments. This approach has proved partially successful, but difficult to accomplish due to the variability of rainfall and drought tolerance.

There has not been a great deal of attention given to separating productivity under drought, which is important for cultivated plants, from survival mechanisms, particularly for woody species. Species or cultivars more tolerant to drought generally differ morphologically and/or physiologically, with mechanisms allowing greater production under limited water supply. These mechanisms involve maximization of water uptake by deep, dense root systems and/or minimization of water loss by stomatal closure and reduction of leaf area (Kramer and Boyer, 1995). These improve plant water status and particularly turgor maintenance, which may be achieved through osmotic adjustment and/or changes in cell wall elasticity, and is essential for maintaining physiological activity for extended periods of drought (Kramer and Boyer, 1995; Turner, 1997). Thus a complimentary approach to improve plant performance for drought prone regions involves the identification and selection of traits that contribute to drought tolerance or water use efficiency (WUE). However characteristics of these traits are quantitative in nature and the ability to identify specific quantitative genes would also lead to a more powerful means of investigating genetic basis.

Over the last two decades, technological advances in the field of genetic and molecular biology have provided new tools to plant breeders for detailed analysis of genomes. The use of molecular markers in the mapping of traits of agronomic importance holds great promise for speeding the development of improved varieties and also improving our

understanding of physiological and molecular aspects behind biological phenomenon (Wang and Paterson, 1994). Genetic maps constructed using molecular markers are of great value in applying genetic technologies in crop improvement such as marker-assisted selection and gene pyramiding (Brar and Dhaliwal, 1997).

Molecular marker technology is a 'near term' for improving drought resistance breeding (O'Toole, 1989). Since drought resistance traits are quantitative in nature, molecular mapping technology and QTL mapping have emerged as powerful tools to accelerate breeding research. Tagging of root traits with molecular markers allows breeders to screen the segregating population, which are derived from contrasting parents for root traits. MAS (marker assisted selection) offer an excellent selection criterion, which is dependent on phenotype, for traits that are difficult to evaluate such as root traits.

The present review briefly discusses selected physiological and morphological traits of robusta and arabica coffee, which may be associated with drought resistance mechanisms, with some implication of molecular approaches for plant breeding. However this review is organized into sections dealing with (i) Climatic factors and requirement, (ii) Crown architecture and dehydration postponement, (iii) Impact and response to drought stress in coffee and (iv) Marker-Assisted Selection for crop improvement.

2.1 Climatic factors and requirements

A brief description of the basic climatic characteristics of the regions where coffee probably evolved is required, in order to ensure a better understanding of the responses of the plant to environmental constraints especially for drought and temperature stress.

The optimum annual mean temperature for robusta coffee ranges from 22 to 26°C (Matiello, 1998), however according to Willson (1999), from 24 to 30°C. High temperatures can be harmful, especially if the air is dry (Coste, 1992). Robusta is much less adaptable to lower temperatures than arabica coffee (15°C). Both leaves and fruits do not withstand temperatures below 5-6°C or long periods at 15°C (Willson, 1999). As altitude relates to temperature, robusta coffee can be grown at mean sea level of 500 to 1,000 m, but even it will be luxuriantly grown at the lower elevations of about 150 metres. However arabica coffee grows best at higher altitudes of 1,000 to 1,500m and is often grown in hilly areas, as in Colombia and Central America. Coffee can also be

grown at lower altitudes further from the equator, unless limited by frost, as is the case of arabica coffee in Brazil.

Rainfall requirements depend on the retention properties of the soil, atmospheric humidity and cloud cover, as well as cultivation practices. The optimum annual rainfall range is 1200-1800 mm for robusta, although it adapts better than arabica to intensive rainfall exceeding 2000 mm (Coste, 1992). A similar range seems to be required for arabica coffee (Alègre, 1959). For both species, a short dry spell, lasting 2-4 months, corresponding to the quiescent growth phase, is important to stimulate flowering (Haarer, 1958). Abundant rainfall throughout the year is often responsible for scattered harvest and low yields. Lack of a dry period can also limit coffee cultivation in lowland tropical regions (Maestri and Barros, 1977).

Air humidity has a significant impact on the vegetative growth of the coffee tree. Robusta successfully grows under high air humidity approaching saturation (80 to 90%), or in less humid sites, provided that the dry season is short. In contrast arabica require 70 to 80% relative humidity.

In coffee plantations the crop yield is usually depressed due to large wind shears and advection. Wind stress may lead to a reduction of leaf area and internodal length of the orthotropic & plagiotropic branches (Caramori *et al.*, 1986), in addition to severely damaging leaves and buds and aggravate shedding of developing flowers and fruits (Camargo, 1985; Matiello *et al.*, 2002). Hot winds increase crop evapotranspiration of the trees increases. Where strong wind is frequent, windbreaks or tree shelter is recommended as both may improve crop performance.

2.2 Crown architecture and dehydration postponement

Stomatal control on transpiration may decrease substantially as the scale increases from leaf to canopy. As the leaf transpires, water vapour tends to humidify the air nearby the leaves, thus decreasing the boundary layer conductance surrounding each leaf and uncoupling the vapour pressure at the leaf surface from that of the bulk air. Tausend *et al.* (2000b), working with three arabica coffee cultivars with contrasting shoot morphologies, showed that regulation of transpiration was governed by divergent hydraulic architecture rather than stomatal physiology. Maximum total transpiration per plant, on a daily basis,

was 13 kg in cv. Typica (conical, relatively open crown with $22.9 \pm 1.7 \text{ m}^2$ leaf area), against 8 kg in cv. Yellow Caturra (flat top, dense crown with $27.5 \pm 3.3 \text{ m}^2$ leaf area). This corroborates some empirical observation that dwarf cultivars with dense crowns (lower boundary layer conductance) are better able to postpone dehydration than cultivars with open crowns, regardless, within given limits, of their leaf areas. At least partially, these findings lend some support for explaining why the use of cultivars with open crowns, even if irrigation is supplemented, often results in crop failure in regions with high evaporative demand, as in north-eastern Brazil (DaMatta, 2004). Therefore, selection of cultivars for drought-prone environments should consider aspects concerning crown architecture.

The comparison of the canopy architecture and the growth and distribution of roots was made in 10-year-old trees of *Hevea brasiliensis* grown in a severely drought-prone area on the west coast of India under rainfed and irrigated conditions. Total root density of a tree per unit volume of soil did not vary significantly between irrigated and rainfed trees. There was also no statistical difference seen in the density of total root as well as in the density of feeder roots at different soil depths and at different distances away from the tree trunk, but the root distribution seems to be different in irrigated and rainfed trees. Most of the feeder roots were found to be present in the top 15 cm of the soil in both treatments. (Devakumar *et al.*, 1999). This could be mainly because of the production of short roots in response to drought and canopy architecture (Gasteller and Vartanin, 1995). This demonstrates dehydration postponement could be attained by high root density and dense canopy architecture.

2.3 Impact and response to drought stress in coffee

From a meteorological point of view, drought refers only to a period in which rainfall fails to keep up with potential evapotranspiration. However, particularly in the tropics, drought episodes are remarkably aggravated by both high solar radiation and high temperature, so drought should be considered as a multidimensional stress (DaMatta, 2003). In fact drought is an environmental factor that produces water deficit in plants, which is initiated when low water potential develops and cell turgor begins to fall below its maximum value (Kozlowski and Pallardy, 1997). In contrast to many other stressful

events (e.g., frosts), stress caused by drought usually does not occur suddenly, but develops slowly and increases in intensity the longer it lasts. Therefore, the time factor plays a crucial role in survival and/or maintenance of productivity under drought conditions.

A major component of differential adaptation to drought among arabica and robusta coffee genotypes appears to be behavioural, and may be governed by rates of water use and/or efficiency of extraction of soil water (Meinzer *et al.*, 1990b; DaMatta *et al.*, 2003; Pinheiro *et al.*, 2005). In this context, comparative studies, both under pot and field conditions, have evidenced that drought-tolerant coffee genotypes are better able to maintain higher Ψ_w and/or RWC than drought-sensitive ones under water-deficit conditions (Boyer, 1965; Nunes, 1976; Josis *et al.*, 1983; Renard and Karamaga, 1984; Meinzer *et al.*, 1990b; DaMatta *et al.*, 2000, 2003; Pinheiro *et al.*, 2005; Praxedes *et al.*, 2006). This characterizes a strategy of dehydration postponement.

The best option for drought tolerance is initially to identify clones with drought resistance traits and develop hybrid population. Such an approach has greater practical relevance because the hybrids can maintain both yield and quality traits. The primary requirement however, is to identify source genotypes with drought tolerance traits. Amongst the many drought resistance mechanisms exploiting the available variability in root characteristics is highly relevant to extract water from deeper layers of soil.

2.3.1 Root characteristics of *Coffea canephora*

Root characteristics and growth play a crucial role in maintaining the water supply to the plant, and drought-adapted plants are often characterized by deep and vigorous root systems. However, predicting the best rooting strategy for a particular crop will depend on the characteristics of the crop's seasonal demand for water, soil depth and chemical and physical properties, as well as the seasonal rainfall distribution. Thus, to breed for a large root system to exploit as much of the soil for water as possible is necessarily the best option.

Most existing research focused on root morphology, growth, root size, activity and spatial distribution. These root traits often been emphasized in relation to drought resistance (Taylor, 1983; Hays *et al.*, 1991; Marcum *et al.*, 1995). However, little is known about

the genetic variations in physiological responses of roots to limited soil moisture. Moreover, extremely limited information is available about physiological factors controlling root mortality under drought stress.

Chiatante *et al* (1999), where, they investigated the effect of water stress on the root system architecture of pine sapling and pea seedling during the first stage development. The data showed that both species are extremely sensitive and that plants arrest their growth immediately during water stress treatment. The root mortality under drought stress was positively correlated with root desiccation, as evidenced by severe leakage of organic solutes from roots in drying soil. In fact the carbohydrate supply to roots was not a contributor to root death during drought stress. The increased or unaffected total nonstructural carbohydrates in both shoots and roots supported this, and the increased C allocation to roots under soil drying conditions. Perhaps this could be due to sensitivity of meristematic tissue situated at root tip, because of the leading role played by this tissue in planning of root architecture.

The coffee root system is highly plastic and can vary according to environmental conditions such as texture, structure, aeration, fertility, reaction of the soil, crop production, management, pests and diseases (Bull 1963a; Cassidy and Kumar 1984; Guiscafne and Gomez 1938; Nutman 1933, Saiz Del Rio *et al.*, 1961). Cuiscafne-Arrillaga and Gomez (1938, 1940 and 1942) concluded from studies in two clay soils in Puerto Rico that: (i). Ninety five per cent of the roots of the coffee trees studied occurred in the top 12 inches of soil; (ii). There is no fixed ratio of shoots to roots in coffee trees (iii). There is a close positive correlation between trunk diameter at the soil line and extent of the root systems and (iv). The coffee tree root system has the shape of an inverted cone, but this can be greatly modified by soil conditions.

Trancoso vaz (1961) found that there is no correlation between soil chemical and physical properties for the root growth in coffee plants, however the root activity markedly varied in the soil profile of depending on the season. This demonstrates growth of the roots was seasonal and it preceded the start of shoot growth for most parts than roots (Huxley *et al.* 1974; Huxley and Turl, 1976).

During hot dry season (January to February) in Kenya, all the vegetative parts of the coffee trees increased very little in dry matter except for the rootlets and are very thinner

than 3mm in diameter (Cannel, 1971a). In fact Carr, (2001) showed, during prolonged drought, relatively high root activity was found at mid-depth near to the trunk but, after the soil profile was rewetted by rain, most root activity occurred in the top soil at quarter row of distance. This demonstrates the root activity and its growth predominantly depending on soil moisture surrounded by the root zone. Therefore, the efficiency of soil water extraction and use are important factors determining crop productivity in semi-arid regions. Generally speaking soil water availability and root water uptake efficiency determines the ability of plants to withdraw water (Eastham *et al.*, 1990). However in millet species vertical deep root penetration would help to avoid the drought stress as a drought avoidance mechanism, in fact it is often restricted by the presence of a hard pan layer. Moreover, drier soil conditions often promote root elongation and increase the degree of branching, allowing the crop to exploit soil water more effectively (Sharma and Ghildyal, 1977).

Robusta coffee trees have shown that their root systems were largely confined to the upper layers of soil; the main lateral roots radiated near the surface of the ground and there was an abundance of soil feeding roots in the superficial soil and in the thin layer of fallen leaves which covers the ground (Thomas, 1944).

The roots are morphologically characterized by following parts,

A taproot, which is often extensively branched, robust, and generally short (0.3-0.5m), but may grow as long as 1m in deep soils. Its main functions are to provide stability and anchorage and to ensure the supply of water to the plant.

A number of secondary roots, which are develop from the taproot and grow vertically downwards. The lighter and more penetrable the soil, the deeper they will develop. Their rôle, together with that of the taproot, is to assist in supplying water to the plant and having 65% of their roots in the top 30 cm of soil and negligible amounts below the 1 mt. levels.

Lateral roots, which are generally numerous, develop, often almost horizontally, and are further extended by a network of rootlets. They explore the superficial and richest layers of the soil. Their role is essentially to provide mineral nutrition to the plant

Drought-adapted plants are often characterized by deep and vigorous root systems. In robusta coffee, by growing four contrasting clones in large containers (120 L), Pinheiro *et al.* (2004) observed that a deeper root system could be associated with better avoidance to soil water limitation in drought tolerant clones than in drought-sensitive ones. Another study has associated drought tolerance with a larger root dry mass, shown by Ramos and Carvalho (1997) working with 29 coffee genotypes. These authors did not find significant genotypic correlations between drought tolerance and production, hence suggesting that these traits were independent of each other. A great limitation of this work resides in assessing the root system using seedlings and, by contrast, assessing production in field-grown adult plants. Moreover, it is not known if the root behaviour at the seedling stage resembles that of the adult coffee tree. In any case, the difficulties in evaluating root systems, the large environmental influences and the complex inheritance of root characteristics hinder the use of these traits in selection programmes, in spite of the obvious positive relationship between root depth, root growth and yield under drought conditions (Medrano *et al.*, 1998). Indeed, drought postponement in clones of robusta coffee has been associated with deeper, but not necessarily larger root (dry mass) systems (Pinheiro *et al.*, 2005). Nevertheless differences between drought-tolerant and drought-sensitive clones in postponing tissue dehydration are even more evident in the field (DaMatta *et al.*, 2000, 2003), where the development of the root system is much less restricted.

Grafting of *C. arabica* cultivars on to *C. canephora* or *C. congensis* increased the development and production of the plants (Fahl and Carelli, 1985; Fahl *et al.*, 1998). The better performance of the grafted plants was partly due to the size and architecture of the root systems; both *C. canephora* and *C. congensis* are tall species, with much more extensive root systems than *C. arabica* (Ramos and Lima, 1980; Fahl *et al.*, 1998). Thus, the use of stocks with more highly developed root systems capable of absorbing a greater amount of water from the soil could be advantageous, in allowing greater stomatal opening and, consequently, higher photosynthetic rates. Thus the grafting of *C. arabica* on to *C. canephora* in addition to considerably increasing the ratio of root: shoot (Fahl *et al.*, 2001 and Fahl *et al.*, 1998), allowed for the exploration of a greater volume and depth of soil, and consequently a greater absorption of water during the drier periods. This

indicates that stomatal aperture and water status of the leaves of the grafted plants were controlled by the more efficient *C. canephora* root system.

Bhat (2002) and Awati (2004) noticed, the significant genetic variability in root characteristics like total root biomass, root to shoot ratio and total dry matter in arabica & robusta coffee accessions. Similarly strong positive association was noticed between root weight and total dry matter under both stress and non-stress conditions signifying the importance of root trait. Also, strong positive association between root weight and total transpiration signified the importance of deeper root system in harnessing the water from deeper layers in drier environments. Thus deeper root system as an adaptive strategy plays a major role in sustaining growth and productivity in perennial crops. This furthermore signifies the root growth and transpiration rate importance under moisture stress conditions.

The root resistance to water transport in the soil-plant pathway seems to be greater in arabica coffee than in *C. canephora* cv. Apoatã (Brunini and Angelocci, 1998; Fahl *et al.*, 2001). Although a contentious issue (see Rena and DaMatta, 2002), Apoatã also appears to have a much more developed root system than arabica cultivars (Ramos and Lima, 1980), which would enable it to increase access to soil water and, as a consequence, to maintain a favourable internal water status. Indeed, *C. arabica* cv. Catuaí grafted onto Apoatã showed considerable increases in root to shoot ratio (Fahl *et al.*, 1998) in addition to having remarkably greater gas-exchange rates than the non-grafted Catuaí plants (*e.g.*, g_s was almost three times larger in both grafted Catuaí and non-grafted Apoatã), as shown by Fahl *et al.* (2001) in field experiments conducted during the dry season in Brazil.

2.3.2 Root to shoot characteristics

Of the different mechanisms of drought resistance, one of the common responses to water stress is the general increase in root to shoot ratio. This is an integral part of the plants and overall strategy to adapt to the perturbation in the soil environment. The reasons for this and the various ways by which the root to shoot ratio can increase have been extensively studied (Jordan and Miller, 1980; Passioura, 1982; Sharp and Davies, 1989).

Plant shoot and root attributes observed at an early stage of growth are related to drought tolerance through dehydration avoidance, a mechanism that allows crop plants to thrive

in water-limited environments. Shoot traits (especially early growth vigor, rapid ground coverage, leaf properties, etc.) are associated with drought tolerance. Likewise, root characteristics (e.g., root size, morphology, depth, length, density, hydraulic conductance), as well as other functions, determine and meet the transpiration demands of the plant (Passioura 1982). Vigorous root development is important for seedling survival under conditions under which the soil surface dries rapidly whilst sufficient soil moisture remains available in deeper zones.

When water availability is limited, root growth is generally less inhibited than shoot growth. This character is thought to be adaptive, where by the roots continue to explore the soil for water while the inhibition of shoot growth, together with stomatal closure, restrict transpiration. An important feature of this response of the root system is the ability of some roots to continue elongating at low water potential that inhibit shoot growth completely (Westgate and Boyer, 1985; Spollen *et al.*, 1995) and similar findings were reported by Sharp *et al.* (1988) in maize seedlings.

Sarker *et al.* (2005) observed considerable variation in stem length, stem weight, taproot length, lateral root number, total root length, and total root weight in a set of eight randomly selected lentil genotypes collected from different regions, represents a specific adaptation to the environmental conditions associated with its area of origin. These infer the conditional adjustment of root shoot ratios to environmental conditions. However taproot length and lateral root number is the most important root traits associated with lentil performance under drought conditions in a Mediterranean-type environment.

Matsui and Singh (2003) studied the effects of soil moisture stress of two cowpea varieties, IT96D-604 (drought tolerant) and TVu7778 (drought susceptible) on the length, dry matter and distribution of the roots. They reported the drought tolerant variety showed no variation in root to shoot ratios under well-watered condition, but the significant variation was found in the mild and severe stress conditions. This could be a drought tolerance mechanism by increasing in root dry matter per leaf area (root to shoot ratio) under water stress conditions.

The dwarf coffee plants seemed to be more efficient in shoot and root dry matter accumulation at early stages of growth. However, it was not possible to separate individually normal from dwarf type plants at seedling stage by shoot length, since the

range (minimum to maximum) was about the same for both groups (Ramos and Carvalho, 1997). Higher dry matter yields for dwarf coffee genotypes in relation to normal ones were also observed by Vishveshwara and Raju (1974).

Root and shoot characteristics in coffee were associated with yield and environmental tolerances (Dublin 1968). However Mazzafera and Carvalho (1987) indicated that in coffee yield and field drought tolerance was not associated. High yield genotypes, which were not usually bred for suboptimal environmental conditions, do not necessarily behave as drought tolerant (Fisher and Wood, 1979). Certain plants have fast growing root systems than shoot at seedling stage, as a way to avoid dry soil surfaces and to use subsurface water more efficiently (White and Castilho, 1992).

2.3.3 Water relations

Several investigators have studied water relations of coffee over the last decades, and some reviews have been published on the subject (*e.g.*, Nunes, 1976; Carr, 2001; DaMatta and Rena, 2001, 2002).

Osmotic adjustment (OA), defined as a net increased solute concentration, may be perceived as an important survival mechanism to drought stress (Turner, 1997), in addition to being frequently correlated with yield stability in dry environments (Blum, 1997). The osmotic adjustment is not a general trait observed in coffee under drought conditions (Golberg *et al.*, 1984; DaMatta 2004b). Therefore, under drought conditions this mechanism, generally been associated with maintenance of gas exchange in many plant species (Turner, 1997), could hardly explain the stomatal sensitivity of coffee to drought.

Since coffee is a shade loving plant, studies have indicated that g_s is greater in the shade than in sunlight coffee leaves (*e.g.*, Kumar and Tieszen, 1980a; Fanjul *et al.*, 1985; Gutiérrez *et al.*, 1994), particularly throughout the morning. However, this behaviour has been attributed to the sensitivity of the stomata to leaf-to-air vapour pressure deficit (D) rather than to solar radiation (Nunes, 1988; Gutiérrez *et al.*, 1994; Carr, 2001; DaMatta, 2004a), hence maintenance of stomatal conductance by keeping more water content in leaf as an avoidance mechanism in coffee. Therefore Coffee retains high leaf relative water content (RWC) under dehydrating conditions, being considered as water saving

rather than a dehydrating tolerant species (Nunes, 1976; Josis *et al.*, 1983; DaMatta *et al.*, 1993, 2002a). A small water loss should therefore cause a shift in turgor so that leaves tend to maintain a high RWC to retain a high symplast volume. Thus it appears that under water deficit the maintenance of a high RWC is more important than osmotic adjustment (OA).

In some species, accumulation of compatible solutes under dehydration tolerance, such as proline and quaternary ammonium compounds, has been considered as an adaptation to drought stress. In coffee, however, such accumulation does not satisfactorily correlate with drought tolerance in various cultivars of arabica and robusta. In any case, a build-up of those compounds under water deficit conditions appears to be a sensitive indicator of leaf water status (Maestri *et al.*, 1995). According to Mazzafera and Teixeira (1989), proline accumulation in drought-stressed plants of arabica coffee might be more directly related to injury imposed by water limitation rather than being a defense mechanism against drought stress.

Robusta coffee appears to exhibit a poorer stomatal control on transpiration than arabica (DaMatta *et al.*, 1997). However, in some progenies of robusta coffee g_s does not apparently respond, or does so poorly, to changes in evaporative demand (DaMatta *et al.*, 1997a; Pinheiro *et al.*, 2005). There is also circumstantial evidence that stomatal aperture in *C. canephora* cv. Apoatã, presumably promoted by chemical signals from roots, is less responsive to decreasing soil water availability than in arabica (Fahl *et al.*, 2001). Because robusta coffee evolved in a wetter region than arabica, the requirement for efficient stomatal control of E would thus be less important for robusta than for arabica coffee (DaMatta, 2003). Considered together, these responses suggest that robusta has a lower stomatal sensitivity to evaporative demand, which leads to a less efficient control of E . This might explain, at least partially, why robusta is more responsive to irrigation than arabica coffee (DaMatta, 2004a,b).

In field-grown arabica coffee, Wormer (1965) demonstrated that applications of N fertilizers increased aperture of stomata without affecting their frequency and size. Segura (1989) found in arabica seedlings that urea sprays increased E , presumably due to increased g_s , which was accompanied by a reduction in water potential. Tesha and Kumar (1978), from field experiments with arabica coffee conducted in Kenya, showed

that E at the leaf level increased with increasing N status under an adequate water supply, and decreased markedly as soil water became less available. Such behaviour obviously is of particular interest in relation to coffee cropping since it would allow minimization of water loss under drought while maximizing carbon gain at the expense of water loss under high soil moisture.

In arabica coffee, hydraulic conductance was found to be positively correlated with total daily transpiration (Tausend *et al.*, 2000a). Thus genotypes with higher hydraulic conductance should have high rates of water use. This means that they may deplete accessible soil water more rapidly and/ or have a deeper root system than genotypes with lower hydraulic conductance. The strong association of total transpiration with hydraulic conductance might dampen variation in water potential with variation in water availability, which may help to avoid non-stomatal limitation to photosynthesis and xylem cavitations (Tausend *et al.*, 2000a). This would be advantageous with non-limiting soil water or with brief periods of water deficit, but disadvantageous with longer-term drought since a high hydraulic conductivity would hasten the development of severe plant water deficit. Hence, knowledge of the nature of the target environment to which cultivars should be bred is crucial for the success of coffee breeding programmes.

2.3.4 Water use efficiency (WUE)

The efficiency with which plant use water, is expressed as the ratio of biomass produced (g) to the amount of water transpired (kg). In other words, the rate of carbon assimilation and the rate of water loss through transpiration control WUE. However, at a given level of transpiration, differences in WUE can contribute to large differences in crop growth rates among genotypes and species.

Significant increase in WUE was observed in 35 *C. canephora* accessions grown under moisture stress conditions. An average of 3.66 and 4.02 g/kg WUE observed under non-stress and moisture stress conditions respectively. The stress induced increase in WUE was strongly linked to the reduction in cumulative water transpired of stressed plants by regulation of stomatal movement (Awati, 2004). The same trend was noticed in 23 arabica accessions (Bhat, 2002) and also in tree species like cashew (Anil Koushik, 1999 and Raju, 2001).

2.3.5 Carbon isotope discrimination

Measurements of the stable carbon isotope discrimination (Δ) of leaf dry matter can provide a useful long-term indicator of WUE (Farquhar *et al.*, 1989). As both diffusion and carboxylation of $^{13}\text{CO}_2$ are slower than those of $^{12}\text{CO}_2$, a higher proportion of ^{12}C is to be expected in plant tissues relative to that occurring in the ambient air. Since Δ is strongly correlated with the ratio of internal to ambient CO_2 concentration (Farquhar *et al.* 1989), it should be then affected by both g_s and A during the period of growth of individual leaves, and these are in turn influenced by environmental, morphological and physiological variables. There apparently is a large genetic variability in Δ , it has a high repeatability and low genotype x environment interaction, thus making Δ very attractive when breeding for high WUE (Richards, 1997). In evergreen woody species, however, leaf Δ integrates phenological rhythms in gas exchange as well as changing environmental conditions. Therefore, restriction of sampling to upper sun leaves, as is often done, may produce results that do not reflect the behaviour of the canopy as a whole (Gutiérrez and Meinzer, 1994a).

There is a considerable genetic variation in Δ in both arabica and robusta coffee (Meinzer *et al.*, 1990a; DaMatta *et al.*, 2000, 2003). Working with five genotypes of arabica, Meinzer *et al.* (1990a) pointed out that a higher long-term WUE (lower Δ) under adequate irrigation resulted from decreased g_s rather than increased A at a given g_s . They also showed that genotypes with higher Δ under full irrigation had greater soil-to-leaf hydraulic conductance, depleting soil water more rapidly and experiencing symptoms of physiological stress earlier when water was withheld, thus indicating that Δ of well-watered coffee plants could be used to predict genotype performance under drought stress. This is of particular interest since a positive correlation between Δ and crop yield has been shown in arabica coffee (Meinzer *et al.*, 1991). Nonetheless, this means that low Δ would be related to low productivity in wet environments on the one hand, and to some stability of coffee yield in drought-prone environments on the other hand.

2.3.6 Oxidative stress

Primary effects of stresses, such as cold and drought, are often related, causing cellular damage and secondary stresses, *e.g.*, osmotic and oxidative stress, resulting in disruption of osmotic and ionic homeostasis and damage to proteins and membranes. It should be

remembered that in tropical areas both low and high temperatures and water deficit may occur concomitantly in field conditions. As such, it is not unusual that oxidative stress control is of common importance for both of these environmental limitations. Few studies have been undertaken in order to associate oxidative stress and drought tolerance in coffee.

Lima *et al.* (2002), showed, the under rapid drought stress the extent of antioxidant system was more in drought tolerant robusta clone 120 than in the drought-sensitive clone 109A. They also showed that the extent of cellular damage, evaluated *via* lipid peroxidation and electrolyte leakage, was substantially lower in the clone 120 than in the clone 109A. Therefore, drought tolerance in robusta coffee might be at least partially associated with increased activity of antioxidant enzymes (*e.g.*, SOD, APX, catalase), as has been noted in different drought-tolerant species and genotypes (Smirnoff, 1995).

In another study, Pinheiro *et al.* (2004) subjected four clones of robusta (14 and 120, drought-tolerant; 46 and 109A, drought-sensitive) to long-term drought until water potential (Ψ_w) before dawn reached -3.0 MPa. They found that induction of defence mechanisms against oxidative stress triggered by drought was greater in drought-sensitive than in drought-tolerant clones. Since the extent of cellular damage was comparatively similar or smaller in the latter, an increased oxidative process suffered by the sensitive clones should have occurred. However, Pinheiro *et al.* (2004) were unable to distinguish the drought-tolerant clone 14 from the drought-sensitive clones 46 and 109A in terms of defence mechanisms to cope with paraquat-induced oxidative stress, and they then proposed that there would be no overall link between oxidative stress and drought tolerance. Nonetheless, revisiting the results of Pinheiro *et al.* (2004) allow us to infer that the drought-tolerant clone 120, which is able to endure long drought spells with acceptable yields under this condition (but not the tolerant clone 14), showed an enhanced ability to cope with paraquat-induced oxidative stress compared to the other clones evaluated. Nevertheless, if drought progresses slowly, the coffee plants can acclimate to water shortage and, thus, they could minimize the deleterious effects of drought, *e.g.*, by partially maintaining photosynthetic rates (Praxedes *et al.*, 2005b).

This was further reinforced by results of Menezes (2005), who observed that SOD activity in clone 120 was maximum at paraquat concentrations as high as 20 μM , whereas

in the clone 109A such an activity was completely abolished. Therefore, a more efficient antioxidant system might play a decisive role in drought tolerance in coffee, particularly in genotypes that favour production over survival in dry environments. A comparison of the results of Lima *et al.* (2002) and Pinheiro *et al.* (2004) also indicated that, if water deficit proceeds slowly, the coffee plants can acclimate to drought and, as a consequence, they could largely avoid oxidative stress.

With regard to arabica coffee plants, peroxidation of membrane lipids plays a decisive role in the high cold sensitivity of roots, probably linked to a limitation in the functioning of antioxidative systems provoked by a decreased respiratory activity. The latter brings about a low availability of reducing power that in turn leads to the failure of antioxidative enzymes to protect lipid membranes, causing a higher root tissue damage and membrane rigidity (Alonso *et al.*, 1997; Queiroz *et al.*, 1998).

2.4 Marker assisted selection for crop improvement

One of the most promising approaches to select genotypes of plants for drought resistance is to map genetic loci influencing these traits. Identification of molecular markers of root characteristics in a perennial crop like coffee assumes greater significance where these traits cannot be quantified very easily. Molecular markers allow breeders to track genetic loci controlling drought resistance without having to measure the phenotype.

Molecular biology can be aid in identifying and selecting the genes involved in drought resistance trait and their influence on yield. O' Toole (1989) pointed out the value of using RFLP technology as marker and selection tools for physiological, morphological or developmental traits for drought resistance. Marker assisted selection of plant characters is moving rapidly forward with a wide range of marker technologies available now (Quarrie, 1996).

Molecular markers are a powerful means for discerning the genetic basis of traits whose phenotypic variation is ostensibly quantitative and polygenic (Tanksley, 1993). With a molecular genetic map, every marker-flanked segment of the genome can be assayed to determine if variation in a segment is significantly associated with phenotypic variation. If so, those genomic segments are termed quantitative trait loci (QTLs). Marker assisted

selection of desired genomic segments can then be used to complement, or replace, phenotypic measurement and selection (Specht *et al.*, 2001).

Hanson *et al.* (1990) suggested that identifying and mapping molecular markers associated with the ability to penetrate compacted soil layers could be used in breeding programs to develop varieties better adapted to water deficit environments. The application of molecular markers and genetic linkage maps has allowed specific regions of the genome controlling drought related polygenetic traits, called QTLs, to be identified. Key QTLs have been mapped for root morphology, root distribution, drought avoidance, root penetration ability, osmotic adjustment, dehydration tolerance, stomatal conductance, leaf rolling, heading date, cell membrane stability, abscisic acid accumulation and several other physiological traits (Price and Courtois., 1999; Katiyar *et al.*, 2003)

In recent years, the development of molecular markers techniques has allowed new opportunity in plant breeding. The conventional selection among breeding populations of coffee is extremely laborious and time-consuming, and Marker-assisted selection (MAS) could overcome most of the current limitation. Despite its economic and agricultural importance, coffee has not been benefited much from molecular techniques, which have been extensively used for other cash crops. There are few reports regarding use of molecular markers in coffee, which are reviewed and given below:

2.4.1 Genetic diversity of *Coffea* species using RAPD markers

Random amplified polymorphic DNA (RAPD) markers generated by arbitrary primers have been used to detect genetic diversity and selective gene introgression in *Coffea arabica* (Orozco-Castillo *et al.* 1994). The resulting dendrograms from RAPD profiles were consistent with the know history and evolution of *Coffea arabica*. RAPD markers have also been used successfully to analyze the genetic diversity among cultivated and sub-spontaneous accessions of *Coffea Arabica* (Lashermes *et al* 1996). Anthony *et al.* (2001) conducted a study of genetic diversity of wild coffee (*Coffea arabica*) using RAPD markers and possible to separate possible to separate the Ethiopian materials from typical and Bourbon accessions.

One of the greatest problems in *Coffea arabica* breeding is identifying precisely any inbred line, based only on botanical and agronomical descriptors, because of the reduced

genetic variability of the species, close pedigree origin, which results in small phenotypic variation. Recently, molecular markers like, RAPD, AFLP and SSR, were used to characterize the genetic variability of commercially used *Coffea* inbred lines. The observed groups were expected, based on previous knowledge of the genetic origin of evaluated cultivars. For the purpose of cultivar identification, a combination of botanical, agronomic and molecular descriptors could be complemented with gene polymorphism identification. (Maluf *et al.*, 2005)

RAPD markers were used to study the genetic diversity in Tanzanian Arabica coffee collected from five different regions of 10 locations. The genetic distances between provenances were fairly uniform suggesting limited variation and coffee accessions clustered according to geographical locations. The results demonstrate that RAPD markers were able to determine variability in the Tanzanian cultivated *C. arabica* accessions (Masumbuko *et al.* 2002).

Silveira *et al.* (2003) the RAPD techniques associated with restriction digestion of genetic DNA was used to assess the genetic variability for rust disease within and among nine populations of *Coffea arabica*, including six progenies belonging to the Sarchimore germplasm. The association of restriction digestion of DNA with RAPD reaction allowed more assessment of the existing genetic variability (Cardamoni Diniz, *et al.* 2005). It was verified that genotypes, which are more susceptible to leaf rust disease, also exhibit higher genetic distance from the genotypes that maintain a high number of genes for resistance. This could provide a valuable material for coffee breeding. The restriction digestion was proved to be useful tool for genetic characterization and identifying genetic base of cultivated varieties is rather narrow in 14 elite *Coffea arabica* cultivars (Berthaud and Charrier, 1988; Sera *et al.*, 2003)

RAPD markers have been successfully employed by Lashermes *et al.* (1996) to analyze the genetic diversity among the cultivated and sub-spontaneous accessions of *C. arabica*. The narrow genetic base of commercial cultivars was confirmed. A relatively large genetic diversity was observed within the germplasm collection. Results suggested an East-West differentiation in Ethiopia, the primary center of diversification for *C. arabica*. The large heterosis effect reported in inter group hybrids were related to genetic

differentiation. RAPD method appeared to be effective in resolving the genetic variations and in grouping *C. arabica* germplasm.

DNA markers were used by Lashermes *et al.* (1997) to investigate the hybrid origin of the allotetraploid species *C. arabica* and to evaluate its genetic variation. Work on chloroplast genome provided strong support to prove that *C. eugenoides* donated the maternal genome of *C. arabica* while the analysis of the nuclear ribosomal DNA indicated that paternal parent was most likely a species belonging to canephoroid group (*C. canephora*, *C. congensis* and *C. brevipes*).

RAPD markers generated by arbitrary decamers have been successfully employed to detect genetic polymorphism and to distinguish materials originating from Ethiopia and *C. arabica* subgroups *C. arabica* var. *typica* and *C. arabica* var. *bourbon*. RAPD analysis reflected the morphological differences between subgroups and the geographical origin of the coffee material (Orozco-Castillo *et al.*, 1994).

The use of RAPD fragments as genetic markers in *Coffea* was investigated by Lashermes *et al.* (1993). Arbitrary oligonucleotides were used as primers to amplify genomic DNA of different coffee accessions representing major *Coffea* species by PCR. Interspecific variation was easily detected in *C. canephora* and *C. liberica*. RAPD markers appeared to be of high value for characterization analysis and utilization of coffee genetic resources.

Knowledge of genetic variation and the genetic relationship between genotypes is an important consideration for efficient rationalization and utilization of germplasm resources (Russel *et al.* 1997). In view of the wide geographical distribution of robusta coffee, characterisation and evaluation of robusta coffee genepool is necessary for effective crop improvement programmes and also for better conservation and management of genetic resources. The primary analysis of genetic diversity of *C. canephora* was studied by enzymatic polymorphism (Berthaud 1986; Montagnon *et al.* 1992).

Genetic diversity among 40 accessions (*Coffea canephora*) of robusta coffee genepool available in India was determined in comparison with 14 representative samples from a *C. canephora* core collection and three accessions of *C. congensis*, using amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) markers. The results revealed that the accessions from the Indian gene bank tend to show good amount

of genetic diversity and inter accession differentiation (Prakash *et al.*, 2005). Another significant outcome of this study is that, the analysis established the genetic relatedness of *C. congensis* with *C. canephora*. *C. congensis* has been reported to be distributed along the river basins in West and Central Africa and well adapted to wet areas of tropical low lands (Berthaud 1986).

It is widely accepted that *C. arabica* has been derived from natural hybridization between different *Coffea* species, therefore the ISSR analysis was employed in identification of putative parents among eight *Coffea arabica* species. The ISSR markers showed high genetic affinities between *C. arabica* var. *arabica* and five interspecific hybrids, forming a group that was supported as a cluster with a bootstrap of 97% (Ruas *et al.*, 2003). These results are in agreement with previous studies based on RAPD data (Ruas *et al.*, 2000). The results achieved with ISSR markers are in agreement with morphological, molecular and crossability data. Suggesting that ISSR analysis could be successfully applied to study the genetic relationship, deducing putative parents of *C. arabica*, and identifying the species involved in origin of interspecific hybrids.

However these ISSR markers were extensively used in the identification of genetic variation in arabica coffee accessions from Tanzania and finding similarity between the *C. arabica* and diploid coffee species. In fact the results of ISSR markers were complimented with RAPD analysis (Masumbuko *et al.*, 2002). These results provide an essential basis for a more accurate picture of genetic diversity among *Coffea* species that may assist hybrid-breeding programme.

2.4.2 Microsatellites in *Coffea* species

Microsatellites are also known as simple sequence repeats (SSRs), are produced by tandem repetition of sequences from 1 to 6 bp long and constitute highly informative markers. Indeed, they are abundant and are distributed uniformly and randomly in the euchromatin of eukaryotic genomes (Tautz e Renz 1984; Wang *et al.* 1994). Furthermore, they are inherited in a codominant Mendelian manner, are somatically stable and highly polymorphic. The polymorphisms are result of the variation in the number of the repeated monomers.

The cultivated varieties of *Coffea arabica* show a very low level of genetic diversity (Bertraud and Charrier., 1988) due to autogamy and the limited number of original plants

from which the main cultivars were derived. Consequently, there are very few allelic variants, making it difficult to find polymorphisms. Numerous techniques have been applied to study polymorphisms in *C. arabica*, such as RFLP (Lashermes *et al.*, 1996a), RAPD (Orozco-Castillo, 1994, Lashermes *et al.*, 1996b), and AFLP (Lashermes *et al.*, 2000), and positive results have in fact been obtained. Nonetheless, the same techniques applied to other species have provided a greater number of polymorphic loci.

Another approach to studying variability in *C. arabica* and identifying polymorphism is the analysis of microsatellites. These highly polymorphic repeated sequences are very informative molecular markers because they are co-dominant and therefore, in contrast to the abovementioned techniques, enable the heterozygous samples to be distinguished from the homozygous. Therefore microsatellites are powerful tools for following specific genes in assisted cross programmes.

The two genomic libraries of *C. arabica* var. Caturra, enriched in the microsatellites in (ATC)_n or (TG)_n. They are the first genomic libraries of this type constructed for this species. The positive sequences were sequenced and total of 69 (ATC)_n and 180 (TG)_n microsatellites were found. These were checked to see the segregation frequency of these alleles in the F₂ progenies. The result indicates, the (TG)₈ repeat present in the locus, had two alleles in parental lines (113bp and 117bp), where F₂ plant showed the 113bp only, two F₂ plants gave the 117bp allele only and all the others gave both alleles. In this case it is clear that the (TG)₈ repeat locus shows diploid type segregation and similar behaviour has been observed in a further 6 polymorphic loci (Rovelli *et al.* 2000). So this kind of SSR analysis could help to find out the allelic polymorphism in segregating individuals.

Simple sequence repeat markers particularly used in the characterization of the genetic groups within coffee species and identification of introgressed genes in interspecific hybrid for the coffee leaf rust, *Hemileia vastatrix*, coffee berry disease, *Colletotrichum kahawae* and root-knot nematodes, *Meloidogyne* spp. from the diploid species *C. robusta* (Anthony *et al.*, 2002). The result revealed the presence of the introgressed gene from the diploid coffee for a particular disease by SSR markers analysis. In SSR cross amplification and variation within coffee trees study, the SSR markers developed for *C. arabica* were analyzed in six diploid species and results explains the genetic relatedness of self sterile and fertile lines (Poncet *et al.* 2004, Baruah *et al.* 2003). The proportion of

heterozygotic loci in one individual was found to be higher for the self-sterile species *C. canephora* (32.3%) and *C. pseudozanguebariae* (22.6%) than for the self sterile *C. heteocalyx* (7%) and *C. sp.* Moloundou (10%).

Combes *et al.* (2000) reported that *C. arabica* and other related coffee species were characterized by microsatellite markers (. DNA clones from a partial genomic library (*C. arabica* var. Caturra) enriched for (TG)₁₃ motifs (Vascotto *et al.*, 1999) were sequenced. Oligonucleotide primers complementary to flanking regions of identified repeats were designed for 11 sequences using computer program Primer 3. SSR assays revealed that all the 11 primer pairs were successful in amplification of variable length fragments. Only five of the 11-microsatellite loci appeared to be polymorphic in *C. arabica* where as microsatellite loci showed broad range of variability across the accessions of *C. canephora*.

2.4.3 Isozyme markers in coffee

Isoenzyme evidence for inter specific evidence of Piata coffee was given by Medina *et al.* (1995). The tree was a natural hybrid from the fusion of a normal *C. arabica* gamete ($n=22$) and an unreduced gamete ($2n=22$) of *C. deweverei*. Isoenzyme banding patterns for phosphoglucose isomerase, phosphoglucomutase and alcohol dehydrogenase confirmed this inter specific origin.

Root characteristics and molecular polymorphism's associated with resistance to *Pratylenchus coffea* in robusta was reported by Touran *et al.* (1995). The anatomy and total phenol content of the roots and polymorphisms of root proteins and genomic DNA was studied in 6 genotypes of *C. canephora* with susceptible and moderate resistance to the nematode. The genetic variation among the group was evaluated with SDS-PAGE analysis of the protein and the RAPD analysis of susceptible and resistance clones with PCR using 17 random primers. Differences between the groups were found in root anatomy, total polyphenol content, protein and DNA patterns. A specific protein marker of molecular weight 29kDa was found in the resistant group, indicating that resistant clones had specific enzyme as product of DNA associated with resistance.

Until recently, genetic diversity among species or cultivars was determined with morphological or isozyme markers. However, these markers are often unsuitable to

measure genetic variation. The analysis of six isozyme patterns in different *C. arabica* accessions revealed absence of polymorphism contrasting with the high level of morphological variation and suggesting that isozymes are not appropriate for the study of genetic diversity and for *C. arabica* accession identification (Berthaoud and Trouslot, 1977).

2.4.4 Introgressive breeding

Introgressive hybridization (introgression) is genetic modification of one species by another through hybridization and repeated backcrossing. Introgression is important in the evolution of flowering plants. It is also important in plant breeding where a desirable trait can be transferred from wild to crop species.

Molecular analysis of Introgressive breeding in *C. arabica* was reported by Lashermes *et al.* (2000). Nineteen arabica coffee introgression lines (BC_1F_4) and two accessions derived from a spontaneous inter specific cross (Timor hybrid) between *C. arabica* ($2n=4x=44$) and *C. canephora* ($2n=2x=22$) were analyzed for introgression of *C. canephora* genetic material. The Timor hybrid derived genotypes were evaluated by AFLP and compared to 23 accessions of *C. arabica* and 8 accessions of *C. canephora*. Analysis of genetic relationships among the Timor hybrid derived genotypes suggested that introgression was not restricted to chromosome substitution alone but also involved chromosome recombination. Introgressive markers identified were estimated to represent 9 per cent to 29 per cent of the *C. canephora* genome.

Selective gene introgression in coffee was detected by using RAPD markers (Orozco-Castillo *et al.*, 1994). Apart from detecting genetic diversity among and with different species of *Coffea*, species-specific amplification products were also identified. Further amplification products specific to *C. canephora* were identified in *C. arabica* genotypes, Rume Sudan and Catimor 5175. This diagnostic product is therefore indicative of inter specific gene flow in coffee and has biological implications for selective introgressive hybridization in coffee.

Enlarging the genetic base and improvement of arabica cultivars has become a priority for coffee breeders. Like wise, understanding the mechanism of introgression provide new perspective to develop suitable strategies for field selection. The results of Herbera *et al.* (2001) suggested new evidence about the favorable deposition of the arabusta

hybrid (*C. arabica* and *C. canephora*) to the introgression. The investigations on genome interactions between *C. arabica* and other diploid related species with molecular tools represent a first step towards real implementation of marker assisted selection in coffee.

Lashermes *et al.* (2000a), using AFLP (amplified fragment length polymorphism) markers recently estimated that the approximate amounts of introgressed materials in many introgressed arabica lines ranged from 8% to 27% of the *C. canephora* genome. As robusta does not have such a good beverage quality as arabica, it is possible to find introgressed lines with both the desired resistance genes and good beverage quality (Bertrand *et al.*, 2003). Prakash *et al.* (2002) indicated the introgression of *C. liberica* genome associated with leaf rust resistance in to *C. arabica* enhanced the possibility that the genetic variation available in this species or other related species can be harnessed for improving many traits in arabica coffee.

Herrera *et al.* (2002), revealed recombination fractions of *C. canephora* chromosomes segments were estimated for seven marker intervals by using 11 RFLP and 13 microsatellites and compared with the recombination fractions previously observed in *C. canephora* for the equivalent marker intervals. The recombination in the (*C. arabica* x *C. canephora* 4x) hybrid is not significantly restricted by the genetic differentiation between chromosomes belonging to the different genomes. The hybrid (*C. arabica* x *C. canephora* 4x) appeared particularly favourable to inter genomic recombination events and gene introgressions. The *C. canephora* alleles indicating a severe counter selection against the introgression of genetic material from *C. canephora* into *C. arabica* by way of triploid hybrids. However, introgressants displaying desirable traits such as a high resistance to leaf rust were obtained (Herrera *et al.*, 2002).

2.4.5 Segregation of markers in F₁ Mapping population

F₁ population was established from a cross between *Litchi chinensis* Sonn. cultivars Wuye and Luhebao using the pseudotestcross format. Twenty-nine 10-mer primers were screened out of 75 primers and 294 RAPD markers were amplified from 68 F₁ seedlings. Of these 53 polymorphic markers, 36 segregated at a ratio of 1:1 (band present; band absent). Among these 36 markers, 21 were present only in 'Wuye' and 15 only in 'Luhebao'; 17 markers segregated at a ratio of 3:1 (band present: band absent) and 232

markers did not segregate in F_1 (the parent genotypes may be $AA \times AA$, $AA \times Aa$, $Aa \times AA$, $aa \times AA$ or $AA \times aa$). Additionally, 7 markers deviated from Mendelian Law and two showed abnormal segregation patterns. Of the 146 markers present in two parents, 123 bands (84.2%) did not segregate and 17 bands (11.6%) segregated normally (3:1). Of the 148 markers present in only a single parent, 14.2% were in 'Wuye' and 10.2% in 'Luhebao'. These results provided the necessary data to construct a genetic linkage map of litchi (Ding *et al.*, 2001). In Pineapple, using F_1 mapping population the genetic map was constructed by RAPD, AFLP and ISSR markers (Carlier *et al.*, 2006).

Segregating markers are useful in generating linkage maps but at least 110 such markers are required [to get at least 10 marker per chromosome ($n=11$)] to construct primary linkage maps in coffee. Bhat (2002) reported only 33 segregating RAPD markers in coffee (*C. arabica*) even by screening 400 random primers. Twenty-seven out of 33 bands segregated in the expected ratios. Out of 33 segregating markers eleven markers were monomorphic between parents and segregated in the mapping population due to heterozygous nature of marker loci in parents. The remaining twenty-two bands were polymorphic between parents and segregated in the mapping population.

2.4.6 Molecular linkage map in coffee

The first *Coffea* interspecific linkage map was based on a backcross progeny [(*C. pseudozanguebariae* (PSE) 3 *C. liberica* var. *Dewevrei* (DEW)) 3 DEW] (Ky *et al.* 2000). The two parental species are diploid with the same chromosome number ($2n \frac{1}{4} 2x \frac{1}{4} 22$). A special strategy was applied, which involved mapping of AFLP bands specific to PSE species relative to DEW. This linkage map comprised 181 loci distributed over 14 linkage groups covering 1144 cM. Although these markers were PSE specific, they were distributed throughout the genome, which is a common feature on AFLP plant linkage maps.

A linkage map for coffee (*C. canephora*) totaling 1402 cM has been developed on the basis of the population of doubled haploids (Pillard *et al.*, 1996). Both RFLP and PCR based markers were used to construct 15 linkage groups. Coffee genomic and cDNA clones provided the source of probes. Totally 47 RFLP and 100 RAPD loci have been placed on the linkage map. A rather low polymorphism rate (18% for RFLP markers and

29 per cent for RAPD markers) was detected. Only 81 per cent of RAPD markers and 85 per cent RFLP markers fit in a 1:1 ratio ($p < 0.01$). The availability of a molecular linkage map has many implications for future development of the genetics and breeding of coffee. Based on the procedure proposed by Hulbert *et al.* (1988), the *C. canephora* genome is 1400 cM. Since the haploid nuclear DNA size of *C. canephora* is reported to be 800Mb (Cros *et al.* 1995).

While working with two complementary segregating plant populations of *Coffea canephora* L., one population comprised 92 double haploids derived from female gametes, while the other population was a test cross consisting of 44 individuals derived from male gametes showed the effect of segregation distortion and analysis of recombination rate in male and female meioses (Lashermes *et al.*, 2001). Use of the two-point map distance estimate corrected for segregation distortion and resulted in a reduction of linkage groups 5 and 9 of 29 and 25%, respectively. The frequency of recombination detected in both populations was very similar at each interval.

Inheritance and genetic mapping of self-incompatibility in *C. canephora* was reported by Lashermes *et al.* (1996). Cross compatibility behavior of doubled haploid and hybrid genotypes were established using both phenotypic bioassay and *in-situ* seed set examination. The crossing behavior, distribution observed within DHs derived from clone IF200 confirmed that self-incompatibility in *C. canephora* is gametophytic self-incompatibility system controlled by a single locus (s-locus). Molecular marker linkage analysis showed that single locus is associated with an RFLP marker on linkage group 9. The availability of linked DNA marker facilitated the genetic analysis of self-incompatibility in relation to coffee breeding programs.

Pearl *et al.*, (2004) AFLPs to construct a genetic linkage map on a pseudo-F₂ population of arabica coffee (*Coffea arabica* L.) derived from a cross between the cultivars Mokka hybrid and Catimor. Sixty trees from this population were selected on the basis of plant height distribution to construct a linkage map. The total length of the map was 1,802.8 cM, with an average distance of 10.2 cM between adjacent markers. This genetic map will serve as the framework for mapping QTL controlling source-sink traits in the same population.

Available linkage maps for *Coffea* species are based on random amplified polymorphic DNA (RAPD), RFLP, AFLP, and microsatellite markers (Coulibaly *et al.* 2003; Ky *et al.* 2000; Lashermes *et al.* 2001; Paillard *et al.* 1996). However, no RFLP markers were common to the different maps and only a few microsatellites have been mapped so far.

2.4.7 Association of trait of interest with molecular markers

RAPD bands linked to trait of interest could be easily identified using two pooled DNA samples: one from the individuals that express the trait, other from individuals that do not. Any polymorphism between the two pools should be linked to the trait. Markers thus identified could be confirmed by mapping in a segregating population. This bulk segregant analysis is now widely used for mapping simple traits with RAPD markers (Rafalski and Tingey, 1993)

Identification of molecular markers that are linked to traits of interest is facilitated by the availability of highly saturated linkage maps. Identification of genetic markers linked to genes that contribute to quantitative characteristics (like yield) is more complicated. Several genes, the expression of which is influenced by environmental factors, encoded many agronomic traits of interest. Very large genetic experiments are required to map these traits with statistical significance. Usually only a subset of the loci of interest may be mapped in any one experimental cross, so that multiple mapping experiments are needed.

Bhat (2002) identified association of RAPD markers with important morphological and physiological traits in F1 progenies of controlled cross between Tafari-kela and S-10 accessions of coffee. He reported co segregation of RAPD marker K19₈₅₀ with the genomic region controlling $\Delta 13C$ and plant stature. Similarly the marker J15₁₀₀₀ and N17₉₅₀ co segregated with QTL controlling carbon isotope discrimination. The marker K8400 showed co-segregation with QTL controlling root length. The marker P5₇₀₀ linked to plant height. The marker Q11₁₈₀₀ was found co-segregating with QTL governing specific leaf weight and QTL controlling water use efficiency and the marker H2₈₀₀ showed co-segregation with mean transpiration rate and net assimilation rate. The SSR marker CM37₁₅₀ showed association with QTL controlling Net assimilation rate.

Similarly CM72₁₉₇ resides near the vicinity of the QTL controlling water use efficiency (Pradeep, 2003).

Single marker analysis was carried out to associate the markers with trait of our interest. Some of the markers were found associated with physiological, morphological and gas exchange parameters in mapping population. The multiple markers viz., OPL18₁₀₀₀, OPM11₁₈₀₀ and OPO16₄₅₀ co-segregated with QTL controlling the $\Delta^{13}\text{C}$. Markers such as OPR4₇₅₀ and OPO6₁₇₅₀ are co-segregated with QTL controlling of WUE. The markers indicated significantly the phenotypic variation in population (Awati, 2004)

An important follow-up of any study is the validation of putative QTL study is the validation of the putative QTLs. Ideally such QTLs should be associated with a phenotypic value, especially in target production environment (TPE) in such a way that breeding gains can be predicted through the introduction of the QTLs into elite varieties (Tuberosa *et al.*, 2002). The potential impact of root trait QTLs on drought tolerance was studied by evaluating IR64 root introgression lines (Shen *et al.*, 2001) under water stress in TPE.

2.4.8 Characterized Amplified Region (SCAR) markers

More recently, SCAR DNA analysis was developed to produce more specific and reproducible results (Paran and Michelmore, 1993; Jung *et al.*, 1999). SCAR markers are created by using a longer primer (extended sequence of a RAPD primer) that has a specific sequence of approximately 20 bases. By increasing the specificity, the results are less sensitive to changes in reaction conditions and more reproducible (Hernandez *et al.*, 1999). Reliable SCAR markers have already been successfully derived from RAPD fragments in *Lettuce*, *Vicia* and *Triticum* (Paran and Michelmore, 1993; Vidal *et al.*, 2000; Hernandez *et al.*, 1999).

The root system plays an important role in regulation of water uptake from deeper soil layers. Root characteristics are cumbersome to evaluate when compared to above ground characters. So the DNA based molecular markers circumvent this problem by amplifying specific sized band for deep rooted and shallow rooted cultivars. A sequence characterized amplified region (SCAR) marker BH14 and a rice microsatellite marker RM 201 were validated in diverse accessions of rice having a variation with root length.

These two root length specific markers identified deep rooted and shallow rooted diverse rice varieties. They showed statistically significant association with maximum root length (Chaitra, *et al.*, 2006). Tagging of marker for any specific trait, which is conferring specific characters especially helps in generating tools for marker-assisted selection, which in turn helps in accelerating the crop improvement.

Root-knot associated RAPD converted SCAR markers, specific for the coffee has been developed. These SCAR markers enable the unambiguous identification of root-knot nematodes coffee (Randig *et al.*, 2002). Therefore, it is concluded that the method developed here has potential for application in routine diagnostic procedures.

The 14 SCAR primer sets derived from *Coffea pseudozanguebariae* (PSE)) AFLP bands were assessed for their ability to amplify homologous sequences in nine *Coffea* species and in *A. thaliana*. SCAR primers would be transferable to related species, mainly when conserved sequences are used for primer design. In contrast to SCAR markers derived from coding sequences such as cDNA RFLP clones, primer-binding sites are less likely to be conserved across species for SCAR markers derived from AFLP. In this context, transferability results obtained are relatively favorable, since any *Coffea* species was amplified by a minimum of 58% primer pairs (Poncet *et al.*, 2005). However twelve primer pairs could amplify across *Coffea* species and one even amplified *A. thaliana*. In this study, the amplification patterns obtained for *C. arabica* were a juxtaposition of patterns of the putative diploid parental species was noticed.

Materials and methods

III. MATERIALS AND METHODS

The major emphasis in the present investigation was molecular characterization of mapping populations with an objective to identify the putative markers associated with a few physiological traits in *Coffea canephora*. In addition to this, attempt was made to develop locus specific SCAR markers for a particular trait and further validate it in the other phenotypically characterized population.

In an earlier study mapping population of two contrasting parents, L1 valley and S.3334 with low and high root characteristics respectively was developed at Central Coffee Research Institute Balehonnur. These parents were identified after initial evaluation of 31 *Coffea canephora* accessions for root characteristics. The mapping population was phenotyped for several morphological and physiological characters such as root traits, total dry matter, root to shoot ratio, cumulative water transpired, and water use efficiency, *etc.* Initially molecular characterization was done using twenty-one informative RAPD primers and based on the association study, putative markers were identified which were related to root traits, WUE *etc* (Awati 2004). However, markers explaining high phenotypic variance for root and other associated physiological traits were not identified with earlier RAPD markers screened. The aim of the present investigation was to further screening with some more informative RAPD and SSR primers and to study their association for the traits. The materials and methods used in this study are explained in this chapter.

Experiment 1:

Development of microsatellite and locus specific SCAR markers

1. Development of SSR markers from *Coffea canephora* genomic DNA
2. Study of allelic polymorphism for developed SSR markers in coffee species
3. Screening the parental lines with RAPD and SSR primers, to identify the polymorphic markers
4. Study of segregated polymorphic markers in the mapping population
5. Association of identified markers with physiological traits
6. Development of sequence characterized amplified region (SCAR) markers for root trait.

Experiment-2

Validation of SCAR markers in the trait specific mapping population

1. Assessment of genetic variability in root traits, WUE and associated physiological traits in *Coffea canephora* accessions
2. Selection of contrasting genotypes for root traits and developing the mapping population.
3. Validation of SCAR marker in the developed mapping population.

3.1 Experiment I

3.1.1 Development of SSR markers from *Coffea canephora* genomic DNA

In the recent years, microsatellites have been recognized as one of the most popular molecular markers with various applications in varied fields. High polymorphism and the relative ease of scoring represent the two major features that make microsatellites of large interest for many genetic studies. The present objective is to develop SSR markers from the genomic DNA. As a first step DNA isolation was done from the S.3334 *Coffea canephora* accession.

3.1.1a The steps followed in the microsatellite isolation

- I. DNA isolation and restriction digestion
- II. Linker ligation to DNA fragments
- III. Enrichment of microsatellite repeats
- IV. Capturing of repeat regions from enrichment PCR product
- V. Cloning and sequencing of repeat containing DNA fragments
- VI. Primer designing to specific repeats

3.1.1b Sample preparation

Freshly matured healthy coffee leaves were collected, dried at 35 ° C in an oven for 15-20 hours and stored in sealed polythene bags. Prior to DNA extraction, the dried leaves were powdered and sieved to obtain fine powder.

3.1.1c DNA Extraction

The method of Porebski *et al.* (1997) which was modified by Bhat *et al.* (2002) was followed for DNA extraction from dried leaves as described below:

Lyophilized leaf powder (0.2g) + 700 μ l of Extraction buffer [20 mM EDTA (pH8.0), 100 mM Tris-Base (pH 8.0), 1.4 M NaCl, 3 % CTAB (Cetyl-trimethyl ammonium bromide), 3 % polyvinyl pyrrolidone, 1 % β -mercaptoethanol]

↓
Incubated at 60-65°C for 30min

↓
After the sample reached to room temperature, ½ volume of Chloroform: iso-amyl alcohol (24:1) was added and centrifuged at 3000 rpm for 20min

↓
To the supernatant, equal volumes of Chloroform: Iso-amyl alcohol (24:1) was added and this step was repeated thrice

↓
To the supernatant, 0.7 volume of propanol was added and kept at -20°C for 2hr for DNA precipitation

↓
DNA pellet was collected by centrifugation at 3000rpm for 20 min and the pellet was dissolved with 250 μ l TE buffer [10 mM Tris + 1 mM EDTA, (pH 8.0)], and kept for RNAase treatment at 37°C for 1hr

↓
To this, equal volumes of phenol: chloroform (1:1) was added and centrifuged at 3000rpm for 20 min

↓
To the supernatant, Chloroform: isoamyl alcohol (24:1) was added and centrifuged at 3000rpm for 20 min

↓
Two volumes of ethanol was added to the collected supernatant and kept in -20°C for DNA precipitation

↓
DNA pellet was collected after centrifugation at 3000rpm for 20 min and 250 μ l TE buffer was added, stored at -80°C (as stock)

3.1.1d DNA purity assessment

The purity of genomic DNA was quantified by Nanodrop DNA quantifier (JH, Bio), measured the absorbance ratio of 260/280 nm. Pure DNA preparations showed a ratio of 1.8 to 2.0.

3.1.1e Restriction digestion (using *RsaI* or *Hae III*)

The isolated genomic DNA (4 µg) was restrict digested with *RsaI* (1 unit) in 1X buffer to get approximately 500bp fragments in 50µl reaction mixture at 37°C overnight. To check the DNA digestion, an aliquot (4-5µl) of the digested sample was electrophoresed on 1.2% agarose gel with a 100bp ladder.

3.1.1f Linker ligation to DNA fragments (super SNX linker ligation)

The aim here is to ligate a double stranded linker on to each end of each digested DNA fragment, so that the linker provides the primer binding site for subsequent PCR steps. They as well provide sites to ease cloning of the fragment into the vectors used subsequently. The linker is therefore compatible with the restriction sites in the vectors multiple cloning site (MCS). The super SNX also incorporates a GTTT pigtail to facilitate non template 'A' addition by *Taq* DNA polymerase during PCR which can be used for T/A cloning (pTZ57R/T). The linkers used are given below:

Super SNX24 forward- 5' GTTTAAGGCCTAGCTAGCAGAATC

Super SNX24+4p reverse- 5' pGATTCTGCTAGCTAGGCCTTAAACAAAA

(a) Preparation of double strand super SNX linkers

The double stranded linkers were made by heating equimolar concentration of both forward and reverse linkers at 95°C in presence of 100mM NaCl, and allowed to cool gradually to room temperature. These double stranded linkers were ligated to *RsaI* digested genomic DNA. This ligation reaction mixture contained 4 µg of digested DNA, 70µM of linkers in presence of 5µM ATP, 10 units of DNA ligase, 1 unit of *XmnI* and 1 X buffer. Finally the ligation was carried out at 16°C overnight.

(b) PCR of the linker ligated DNA:

To ensure that the linker ligation has worked, PCR of linker ligation was performed in a 25µl reaction as follows: 2µl of ligated DNA mix as template, 10µM of SNX 24 forward primer, 150µM of dNTPs, 2mM MgCl₂, 1 unit of *Taq* DNA polymerase and 25 µg/ ml of BSA. All reactions were carried out in 1x PCR buffer concentration. The PCR cycling conditions were as follows: 95°C (initial denaturation) for 2 min followed by 20 cycles of

denaturation at 95°C for 20sec, annealing at 60°C for 20sec. and extension at 72°C for 1 min 30sec. Approximately 5µl of PCR product was electrophoresed on 1.2% agarose gel to check if the linker ligation was successful, using 100bp ladder as a marker.

3.1.1g Enrichment for microsatellite containing genomic DNA fragments

The aim here is to remove the unwanted DNA fragments and capture the repeat DNA fragments (microsatellites) using biotinylated complementary microsatellites oligos (probes). The biotinylated oligos used for enrichment were: (CA)₁₇, (AG)₁₆, (AGC)₈, (AGG)₈, (AAC)₁₄, (ATC)₁₄, (AAG)₁₄, (ACGC)₅, and (ACCT)₈.

The materials required for this step are;

- a) Streptavidin beads (washed twice in tris EDTA buffer and twice in 1x hybridization solution).
- b) 2x hybridization solution (2x hyb) - 12x SSC, 0.2% SDS (warmed)
- c) 1x hybridization solution (1x hyb) – 6x SSC, 0.1%SDS (warmed)
- d) Washing solution- 2x SSC, 0.1% SDS (warmed)
1x SSC, 0.1% SDS (warmed)
- e) Biotinylated oligos (individual or mixture of 3' biotinylated oligos)

*(SSC-Sodium Saline Citrate, SDS-Sodium Dodecyle Sulphate)

The enrichment of microsatellites was carried out in final volume of 50µl containing 25 µl 2x hybridization solution, 10 µl equimolar biotinylated microsatellite oligos and 2µg of linker ligated DNA. The PCR amplification was carried out using an Eppendorf Master Cycler gradient. The programme consisted of the following steps; 99 cycles of amplification follows 95°C/5 min, 70°C/5 sec, 68.8°C/5 sec, 68.6°C/5 sec with step down of 0.2°C for every 5 sec until it reaches 50°C and maintained at 50°C for 10 min, followed by 20 cycles consisting of 49.5°C/5 sec with step down of 0.5°C every 5 sec until it reaches 40°C/5 sec and finally held at 15°C. The touch down PCR conditions facilitate the microsatellite probes to hybridize with complimentary DNA repeat fragments (i.e., expectantly long prefect repeats) when the reaction mixture is at or near the microsatellite probes melting temperature.

3.1.1h Capturing of repeat regions from enrichment PCR product

After enrichment reaction, to capture microsatellite repeats the following method was performed.

- 1) The streptavidin beads were washed twice with Tris-EDTA (TE) buffer and twice in the hybridization solution as follows, 2x hyb. (12x SSC, 0.2% SDS), 1x hyb (6x SSC, 0.1% SDS) and washing solution (2x SSC, 0.1% SDS, 1x SSC, 0.1% SDS)
- 2) To the enrichment reaction mix, 50µl of washed streptavidine Dynabeads were added and transformed into 1.5 ml eppendorf tube containing 250µl of TE and mixed well.
- 3) These beads were captured under the magnetic particles collecting (MPC) unit and again these beads were washed with TE and 1x hyb solution. Finally these beads were incubated at room temperature after resuspension in 150µl of 1x hyb solution kept on rotator with low speed for 30 min.
- 4) After incubation, the supernatant was removed and beads were collected under the magnetic field. These beads were again washed twice with washing solution at 45°C to 50°C.
- 5) After washing 200µl TE was added, vortexed and incubated at 95°C for 5 min. Then the beads were captured under the magnetic unit and supernatant containing enriched fragments (i.e., “**GOLD**”) were collected in to the new tube.
- 6) To the microsatellite containing supernatant, 22µl of 3M NaOAc (Sodium acetate) and 444 µl of 95% ethanol was added, mixed well and kept on ice for 15 min. then centrifuged at 12,000rpm for 10 min
- 7) Supernatant was discarded and 0.5 ml of 70% ethanol was added, centrifuged for 1 min, again the supernatant was discarded and pellet was resuspended in 25 µl of TE (**Pure Gold**)

3.1.1i PCR recovery of enriched DNA

To recover the enriched pure gold DNA fragments, PCR was carried out in a 25 µl reaction contained 1x PCR buffer, 0.5 µM super SNX Forward primer, 150 µM dNTPs, 2.0 mM MgCl₂, 25 µg/ml BSA, 1 U *Taq* DNA polymerase, and 20 ng of pure gold DNA.

The DNA amplification was carried out using a PTC 100 (MJ Research) thermal cycler, programmed with 3 min at 94°C for initial DNA denaturation, followed by 25 cycles of 94°C for 20 sec, 60°C for 20 sec, 72°C for 1.5 min, final extension at 72°C for 30min.

3.1.1j Cloning and sequencing of repeat containing DNA fragments

(a) Ligation of enriched DNA fragments into pTZ57 R/T vector

Purified PCR product (50ng) was ligated to pTZ57R/T linearized vector (100 ng) using 1U of T4 DNA ligase over night at 16°C in a 10 µl reaction volume, 2.5 µl of ligated mix was used for bacterial transformation in DH5α competent cells (Sambrook *et al.*, 1989). The transformed bacteria were plated on ampicillin selection with X-gal for blue white selection. White colonies were screened by colony PCR using M13 forward and reverse primers (3 µM), 100 µM dNTPs, 2 mM MgCl₂, 1 U of *Taq* DNA polymerase and 1X PCR buffer in 15 µl reaction volume, at annealing temperature of 57°C and final extension at 72°C for 2 min. The PCR products were resolved on 0.8% agarose gel, using 100bp ladder and the inserts ranging from 500-1000bp were again amplified in a 50 µl reaction and purified for sequencing.

The isolation of microsatellites with selective hybridization was schematically represented in Fig 1.

(b) DNA sequencing

The purified PCR products were sequenced with nested M13 forward and reverse primers using ABI prism sequencer (Delhi University, South Campus, New Delhi).

(c) Analysis of the sequence data

The sequences obtained were analyzed for the presence of microsatellite regions using the web-based software –“Tandem Repeat Finder”.

3.1.1k Primer designing and PCR standardization

(a) Primer designing

Clones with >15bp microsatellite region were selected for primer designing using the FastPCR software programme. Primers were designed in the flanking region of microsatellites and synthesized as desalted oligos (SIGMA) PCR standardization was

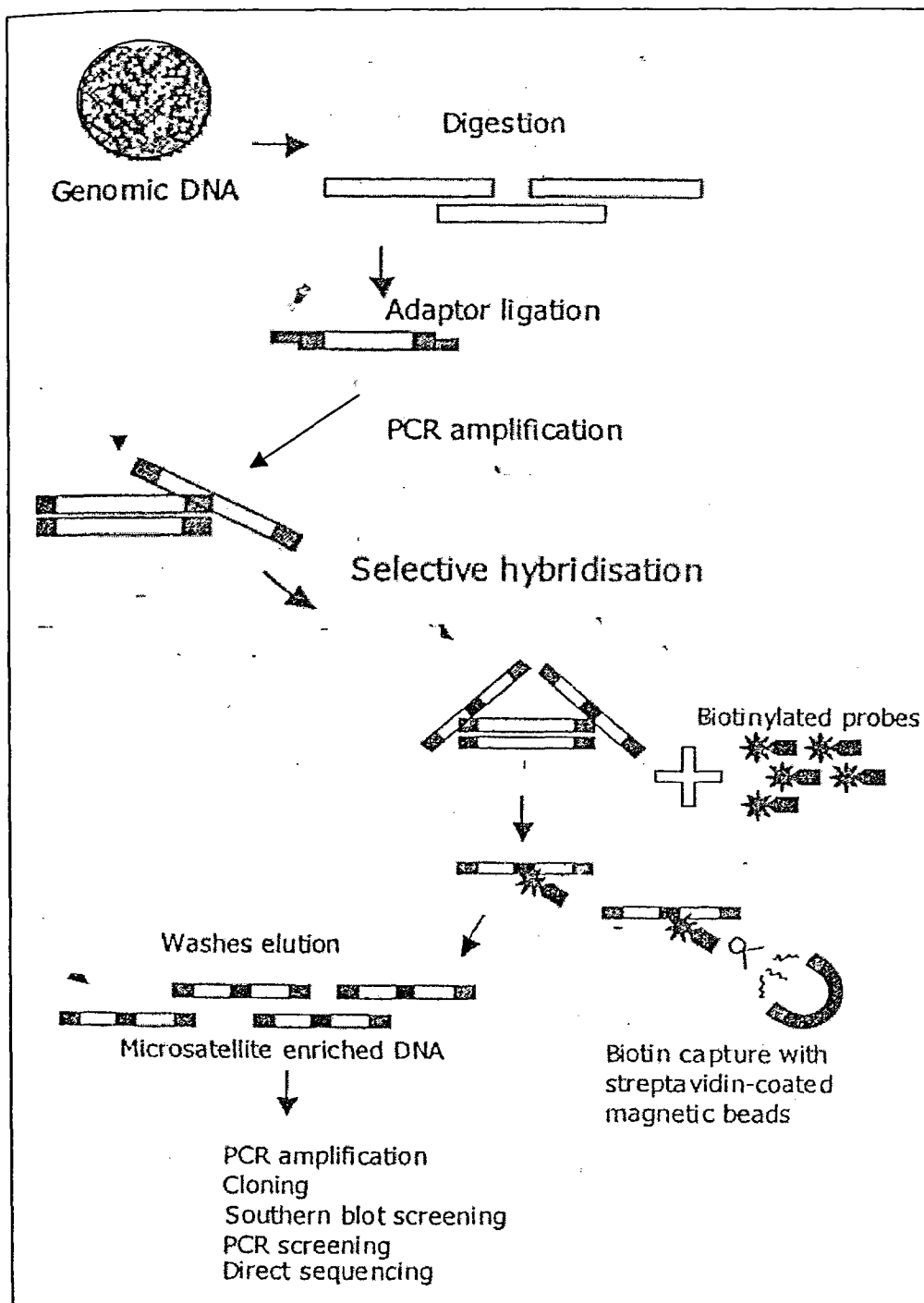


Figure 1 . Schematic representation of selective hybridization

carried out using the genomic DNA as template to arrive at the optimum annealing temperature for each primer synthesized.

(b) Optimization of annealing temperature for microsatellite primers

The PCR amplifications were carried out in 15µl reaction volume containing; genomic DNA (5ng/µl), PCR Buffer (1X), MgCl₂ (2.5mM), 2mM dNTPs, 2µM forward and reverse primers and 1 unit *Taq* DNA Polymerase. Amplification was performed in a Eppendorf Master Cycler Gradient, programmed for initial denaturation at 94⁰ C for five minutes, followed by 35 cycles; each cycle consisting of denaturation at 94⁰ C for 30sec, gradient annealing temperature ($\pm 5^0$ C of oligos T_m) for 30sec, primer extension at 72⁰ C for 1 min and a final extension of 5 min at 72⁰ C. The amplified products were resolved on 3% agarose gel in 1x TAE (40mM Tris-acetate, 1 mM EDTA pH 8.0) buffer at 90 volts for 2 h. the gels were documented using HeroLab Gel Documentation system.

3.1.2 Study of allelic polymorphism for developed SSR markers in coffee species

The genomic DNA of nine coffee species was extracted as described earlier, and amplified with the standardized primers. The PCR amplification and electrophoresis was done as mentioned earlier. The polymorphic loci scoring for its presence (1) or absence (0) was done among the nine coffee species.

3.1.3 Screening of RAPD and SSR primers for polymorphic bands between parents

Total genomic DNA was extracted from frozen-dried leaves of S. 3334 and L1 valley plants using as explained earlier. RAPD and SSR primers were screened to identify the primers generating polymorphic bands; using the genomic DNA of the above two parents.

The contrasting parents (L1 valley x S.3334) for root traits were screened with three hundred (Series from OPK1 to OPZ20) random decamer primers (Operon Technologies Inc). DNA samples (15ng/µl) of 134 mapping individuals were amplified in a 15 µl reaction containing 1x buffer, 2mM dNTPs, 2.5mM MgCl₂, 5 pico moles primer, 1 U *Taq* DNA polymerase, at 94⁰ C/4 min hot start denaturation, followed by 35 cycles of 94⁰ C

for 1min, primer annealing at 38⁰ C for 1 min, extension at 72⁰ C for 2 min and a final extension at 72⁰ C for 8 min. The PCR products were separated on a 1.5% agarose gel at 90 volts for 2h and documented using HeroLab Gel Documentation system.

For SSR analysis, around 55 primers were used to study the polymorphism between parents. The amplification and electrophoresis conditions were followed as explained earlier.

3.1.3a Study of segregating polymorphic band in the mapping population

The polymorphic RAPD bands were visually scored for the presence (1) or absence (0) and in SSR analysis the segregating band from the female parent was scored as 3 and male parent as 1. The heterozygous bands were scored as 2 in all individuals of F1 mapping population. The binary data was used for further statistical analysis

3.1.3b Genotypic interpretation

Based on the segregation of RAPD markers in the mapping population, the putative genotypic interpretation of the parents for the marker locus was made and the Chi-square test was performed (http://www.physics.csbsju.edu/stats/chi-square_form.html).

3.1.3c Association of identified polymorphic markers with physiological traits

Single point analysis (Tanksley *et al.*, 1989) for detecting the association of molecular markers with complex physiological and morphological traits was done using SAS software. To find the amount of variability explained by these markers, regression (R^2) values were worked out by one-way analysis of variance (ANOVA), by general linear model (GLM) procedure. In this analysis, different traits were treated as dependent variable and the various molecular markers as independent variables. A total of 30 different physiological and morphological traits were used to associate with the 85 molecular markers (SAS, 1989).

3.1.4 Development of locus specific Sequence Characterized Amplified Region (SCAR) markers

One of the RAPD bands linked to low root length and low root dry weight locus identified in the mapping population of L1 valley x S.3334 was excised from the gel, purified using perfectprep gel cleanup kit (eppendorf), following the manufacturer's protocol and cloned in to pTZ57R/T vector using T/A cloning kit (MBI Fermentas). White colonies were grown in 3 ml LB medium containing 100µg/ml ampicillin, and the plasmid was isolated with the QIA Prep Spin Miniprep kit (Qiagen). The clone was sequenced by automated sequencer, using M13 universal primers. The sequence information was used to design the forward and reverse primers. The primers were synthesized as desalted oligos from SIGMA.

Primers were standardized for amplification of the band at different gradient temperature to arrive at optimal annealing temperature. The PCR conditions of SCAR reactions were the same as RAPD reactions described earlier.

3.2 EXPERIMENT -: II

3.2.1 Validation of SCAR markers in the trait specific mapping population

3.2.1a Assessment of genetic variability in root traits, WUE and associated physiological traits in *Coffea canephora* accessions:

Gravimetric determination of root, WUE and other associated physiological traits

In the first experiment the accessions were grown in two moisture regimes maintained at 60% FC (moisture stress) and 80% FC (non stress). During experimental period the accessions were characterized for many morphological and physiological traits. However to revalidate the consistent morphological and physiological characteristics, of these accessions the second experiment was conducted at 80% FC. The method followed in two experiments was same and presented below.

3.2.1b Planting material

To identify genotypic variations and for the selection of genotype with contrasting root growth and root to shoot dry weight ratios under non-stress conditions, the methodology followed is given below.

Since, robusta is a cross-pollinated and heterozygous species, to get true to type of plant material, four months old healthy orthotropic shoots with dark-green foliage were collected during the rainy season (June-July). Thirty-five *Coffea canephora* accessions including exotic species were selected for raising the single nodal cuttings at regional coffee research station, Chundale, Kerala. From the selected shoots, 10 cm length single node cuttings were prepared. To prevent fungal infection, cuttings were treated with a fungicide solution by immersing the cuttings for 15 minutes. As a pre-planting treatment, IBA solution at 1000ppm concentration was used as quick-dip application. All the treated cuttings were then inserted vertically into polythene bags containing soil mixture in a proportion of 6: 2:1 (Forest soil: Sand: FYM). These polythene bags were arranged in the propagation trenches and after planting the cuttings; the trench was covered with transparent polythene sheet (Plate-1a). These plants were maintained for one year in the nursery trenches for better root establishment (Anil Kumar, 1994). One year after establishment of suckers/clones at nursery conditions, the selected single nodal cuttings of 35 accessions were then transplanted to the big battery containers (Plate 1b). The accessions used for the investigation are listed in Table 1.

3.2.1c Pot culture studies

(a) Design and layout

Completely randomized design (CRD) was followed, using moisture regimes as main treatment and genotypes as sub treatment.

Moisture regimes: - T1: 80 % field capacity (% FC)

T2: 60 % field capacity (% FC)

Number of genotypes: 35 accessions

Replications: 5

(b) Preparation of pots

Battery containers with a capacity to hold 35 kg of soil were used. Plastic ropes were tied after drilling holes, to facilitate lifting of pots for weighing and maintaining the moisture levels on gravimetric basis. Each container was filled with potting mixture consisting of forest soil, farmyard manure and sand in 6:2:1 proportion. Six pots were maintained in

Table 1. *Coffea canephora* accessions used to study the morphological and physiological characteristics through gravimetric approach

	Accessions
1	S. 8791(JAVA)
2	S. 1932 (MADAGASCAR)
3	S. 1902 (SAIGAN)
4	S. 880 (UGANDA)
5	S. 1979 (UGANDA)
6	S. 3389 (COSTARICA)
7	S. 1589 (IVORYCOST)
8	S. 1481 (GUATEMALA)
9	S. 3400 (COSTARICA)
10	S. 3855 (IVARYCOST)
11	DR-1
12	DR-2
13	DR-3
14	DR-4
15	DR-5
16	DR-6
17	DR-7
18	DR-8
19	DR-9
20	DR-10
21	DR-11
22	DR-12
23	DR-13
24	DR-14
25	DR-15
26	DR-16
27	DR-17
28	DR-18
29	DR-19
30	DR-20
31	SIB-4
32	SIB-7
33	ROBUSTABOLD
34	ROBUSTA HIGH YIELD
35	DWARF CxR

Note:- S, selection; DR, Drought Resistance;



Plate a. Single nodal cuttings in nursery trenches



Plate b. Established single node cuttings in big containers

Plate 1: Establishment of single node cuttings in battery containers. The true to type of single nodal cuttings were planted in polythene bags & were arranged in the propagation trenches (**plate a**). After planting the cuttings, the trench was covered with transparent polythene sheet. One year after establishment of suckers/clones at nursery conditions, the selected single nodal cuttings of 35 accessions were then transplanted to the big battery containers for gravimetric measurements (**Plate b**).

each treatment for each genotype. At the time of planting, recommended dosage of fertilizers was applied.

(c) Treatment imposition

One-year-old clones raised in poly bags were transplanted to battery containers and watered regularly. The containers with *C. canephora* seedlings were placed in an open area under shade net to protect from direct sunlight during daytime. These plants were kept under mobile rain out shelter (ROS) as shown in **Plate-2**, to prevent any external moisture from rain interruption. Whenever required during day and during nights the ROS was drawn over the experimental area, thus the containers were maintained at the specified water regime. This mobile ROS designed and fabricated at ICRISAT, Asia Centre was modified further to improve the efficiency (Udaya Kumar *et al.*, 1998a).

Thirty days after transplanting, all the containers were saturated with water and left over night to bring the soil to 100 per cent field capacity (FC). The drainage holes of the containers were closed using cement. The exposed part of soil in the containers was completely covered with plastic pieces (battery pieces weighing 1 kg were used to cover the soil uniformly for all containers) to prevent the evaporation from the soil surface. The weight of individual containers with battery pieces and plant was recorded with the help of a mobile electronic balance of 150-kg capacity. The balance could be brought to each individual container and the weights of 175 containers were recorded.

All the containers in each genotype were grouped into two sets. One set of containers were maintained at 80% FC and other set of containers at 60% FC. The containers were daily weighed once (between 9.00 to 10.30 am) to record the amount of water loss through transpiration. After weighing, required amount of water was added to bring back the soil moisture status to 60 and 80% FC, respectively.

3.2.1d Computation of pot weight and amount of water needed for maintaining the desired soil moisture regime

Pot weight at 80% FC (w 100) = A+B+Q80

Pot weight at 60% FC (w 100) = A+B+Q60



Plate 2: *Coffea canephora* seedlings under shade net and Rain out shelter (ROS). The 35 *C. canephora* accessions were maintained in 80% FC and 60% FC in 35 kg soil holding battery containers. To prevent excess light the shade net, rain interruption the plants were kept under 70% agro shade net and rain out shelter (ROS).

Where

A= soil dry weight with pot

B= plastic pieces (spread on each pot surface to reduce surface evaporation)

Q 80 = Quantity of water present at 80% FC

Q 60 = Quantity of water present at 60% FC

The required amount of water (PQ) on each day in each pot was calculated as follows,

$$PQ\ 80 = W\ 80 - X\ 80$$

$$PQ\ 60 = W\ 60 - X\ 60$$

Where

X 80 = daily weight of pots at 80% FC

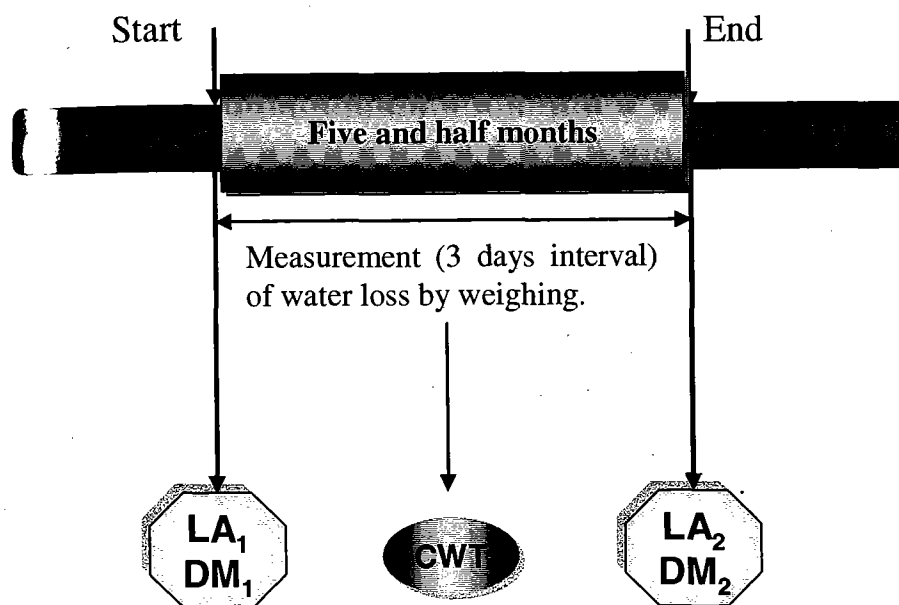
X 60 = daily weight of pots at 60% FC

3.2.1e Measurement of daily evaporation loss from containers

Since there was water loss by evaporation, in spite of the fact that the soil surface was covered with plastic pieces, the daily evaporation was measured with a set of buffer containers (with same amount of soil and plastic pieces, without plants). These containers were also weighed daily for evaporation, and required amount of water was added daily to bring back the containers to respective soil moisture regimes. The cumulative water added to these respective treatments were noted as CWA* and used as a correction for the evaporation loss. This corrected CWA for each container (CWA-CWA*) was taken as the cumulative water transpired (CWT) in each container, during the treatment period of 90 days (Fig 2). The containers were prevented by any external moisture entry using portable rain out shelter (ROS).

3.2.1f Crop management

Prophylactic plant protection measures were taken mainly to control the damage of crop by pests and diseases by spraying protective chemicals periodically as per the recommended package of practices.



$$WUE = (DM_2 - DM_1) / CWT$$

$$NAR = (DM_2 - DM_1) / LAD$$

$$LAD = (LA_1 + LA_2) / 2 \times \text{no. of treatment days}$$

$$MTR = CWT / LAD$$

Fig 2. Schematic representation of the experimental protocol for the gravimetric measurement. The potted plants were weighed once in three days to measure cumulative water transpired (CWT). Using CWT and growth parameters like total biomass (DM), leaf area (LA), leaf area duration (LAD), etc., were used in calculating the water use efficiency (WUE), net assimilation rate (NAR) and mean transpiration rate (MTR).

3.2.1g Observations recorded during treatment period

1. Leaf area (cm²/plant)
2. Leaf dry weight (g/plant)
3. Root dry weight (g/plant)
4. Shoot dry weight (g/plant)
5. Cumulative water added (CWA) (kg/plant)
6. Cumulative water evaporated (CWA*) (kg/pot)

3.2.1h Based on these preliminary data, the following parameters were computed:

1. Total dry matter (g/plant)
2. Root to shoot ratio
3. Leaf area duration (LAD) (dm² days)
4. Net assimilation rate (NAR) (mg/ dm² days)
5. Mean transpiration rate (MTR) (ml/dm² days)
6. Cumulative water transpired (CWT) (kg/plant)
7. Water use efficiency (WUE) (g/kg)

1. Total dry matter accumulated (TDM)

The biomass accumulated during the treatment period (30 to 120 DAS) was computed as the difference in initial and final dry matter and expressed as grams per plant.

TDM = Root dry weight + Shoot dry weight

2. Root to shoot dry weight ratio (RSR)

The ratio of root dry weight to shoot dry weight was calculated at the treatment period.

$$\text{RSR} = \frac{\text{Root dry weight (RDW)}}{\text{Shoot dry weight (SDW)}}$$

3. Cumulative water transpired (CWT)

$$\text{CWT} = \text{CWA} - \text{CWA}^*$$

CWA = Cumulative water added

CWA* = Cumulative water evaporated

4. Leaf area duration (LAD)

It is the functional leaf area available for assimilation during the active growth period.

LAD was calculated using the standard formula given by Hunt, (1982) for container studies.

$$LAD = \frac{(LA1) + (LA2)}{2} \times (t_2 - t_1)$$

LA 1 = Initial leaf area. (dm²)

LA 2 = Final leaf area (dm²)

t₂ - t₁ = Number of treatment period (days)

5. Water use efficiency. (WUE)

Water use efficiency is the amount of dry matter produced per unit amount of water transpired. It was estimated as the ratio of the dry matter accumulated (including roots) during treatment period (30 DAS to 120 DAS) to that of the cumulative water transpired during the same period and expressed as g/kg.

$$WUE = \frac{DM_2 - DM_1}{CWT}$$

Where

DM 1 = Initial dry matter

DMI 2 = Final dry matter

CWT = Cumulative water transpired

6. Net assimilation rate (NAR)

NAR is the amount of biomass produced per unit leaf area per unit time.

$$NAR = \frac{DM_2 - DM_1}{LAD}$$

Where,

DM 1 = Initial dry weight, DM 2 = Final dry weight

LAD = Leaf area duration

7. Mean transpiration rate (MTR)

The ratio of the cumulative water transpired (CWT) and functional leaf area (LAD) is a reflection of the mean transpiration rate and it is expressed as gdm^2 .

$$\text{MTR} = \frac{\text{Cumulative water transpired (CWT)}}{\text{Functional leaf area (LAD)}}$$

The phenotypic data was analyzed by following two factor completely randomized design was used to calculate the critical difference (@ 0.01) with MSTATC software (MSTAT-C, 1991)

3.2.2 Molecular analysis of 35 *Coffea canephora* accessions

Molecular analysis pertaining to the DNA extraction, quantification, purity assessment, and PCR amplifications were carried out as explained earlier.

This study was carried out to assess the genetic diversity of *C. canephora* accessions using RAPD primers. Eighteen informative RAPD primers were amplified in the 35 *C. canephora* accessions following the PCR protocol as explained earlier and the amplified products were resolved on 1.2% agarose gel. The amplified products were used to estimate the genetic diversity using Wards method. The data matrix was converted to dendrogram and three dimensional principal component analyses was carried out.

3.2.2.1 Statistical analysis for estimation of genetic diversity using morphological traits and RAPD markers

(a) Estimating genetic distance by RAPD markers

The binary data scored for the presence (1) or absence (0) from each RAPD primer in all the 35 accessions were analyzed with the computer package called “STATISTICA” (Statsoft 1996). A dendrogram was constructed for both phenotypic and genotypic data using UPGMA (unweighted pair-group method using arithmetic averages) method.

The dissimilarity matrix was developed using Squared Euclidean Distance (SED) that estimated all pairwise differences in the amplification product (Sokal and Sneath, 1973). The genetic distance was computed as follows;

$$\sum_{j=1}^n dj^2 \quad \text{where } dj = (X_{ik} - X_{jk})$$

Where X_{ik} refers to binary code of i^{th} tree for allele “k” and X_{jk} refers to the binary code of the j^{th} tree for allele “k”.

(b) Principal Component Analysis (PCA)

To identify the individuals clustering, PCA was carried out using dissimilarity matrix, STATISTICA software. It calculates mean variance (computed as the average squared deviation of each number from its mean) of all parameters of each accession and groups them into three factors. These factors were classified based on the definite variance range values. Based on the range values, the accessions were classified and clustered.

3.2.2.2 Estimating genetic distance by morphological traits

The morphological and physiological data was used to study the genetic distance. Instead of the binary data, phenotypic data was directly used to calculate the genetic distance. The methodology to calculate the genetic distance and PCA was followed as explained earlier.

3.2.2.3 Correlating the genetic distance estimated by morphological traits and RAPD markers

The distance matrices developed for 35 *Canephora* accessions from morphological traits and RAPD markers were further correlated using the Windows Excel software. The regression (r) values were estimated in order to determine the correlation between the sets of data studied.

3.2.3 Development of trait specific mapping population of *Coffea canephora*

3.2.3.1 Development of the materials for phenotypic, genotypic and molecular marker analysis

(a) Location of the experiment

Experiment was conducted at Regional Coffee Research Station (RCRS), 2004, Chundale.

(b) Crossing

The two accessions with contrasting root characteristics were selected for crossing. One elite accession with good root system as male Madagascar and one accession with inferior root system as female DR-14 were crossed to obtain the mapping population.

(c) Emasculation and pollination

In the female plant, one fully flowered branch was selected. Flowers were opened forcibly when they reached the candle stage and stamens were removed. The collected pollens from the male parent were dusted on the female stigma for effective pollination. To prevent the cross pollination the pollinated flowered branch was covered with cloth bag until seed set.

(d) Seed collection and sowing

After a period of 9 to 10 months, fruits were collected from the female parent (DR-14) and used in the study. The seeds were sown in a nursery bed. It took 48 days for germination. At topi stage, 127 healthy seedlings were transplanted to polybags. Six month-old seedlings were transplanted into carbonized rubber containers to study the physiological traits. The treatments were imposed one month after transplanting.

3.2.3.2 Phenotypic analysis of mapping population

(a) Estimation of variability in physiological traits in the mapping population

The mapping population raised by crossing was used in the present study. Gravimetric technique as explained earlier experiment was adopted to study the phenotypic characteristics and physiological traits in the mapping population. The duration of the experiment was 269 days. An initial observation for root weight and TDM was recorded

The dissimilarity matrix was developed using Squared Euclidean Distance (SED) that estimated all pairwise differences in the amplification product (Sokal and Sneath, 1973). The genetic distance was computed as follows;

$$\sum_{j=1}^n d_j^2 \quad \text{where } d_j = (X_{ik} - X_{jk})$$

Where X_{ik} refers to binary code of i^{th} tree for allele “k” and X_{jk} refers to the binary code of the j^{th} tree for allele “k”.

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3.2.2.3 Correlating the genetic distance estimated by morphological traits and RAPD markers

The distance matrices developed for 35 *Canephora* accessions from morphological traits and RAPD markers were further correlated using the Windows Excel software. The regression (r) values were estimated in order to determine the correlation between the sets of data studied.

on other plants of the same age. Leaf area was measured by length x breadth x leaf area factor (0.65). Since each individual of the F_1 generation is different from one another, replications could not be maintained. This experiment was carried out at only one level of moisture regime i.e., 70% FC. Physiological parameters were recorded and analyzed as explained earlier.

3.2.3.3 Analyzing SCAR markers in the mapping population

Each of the designated SCAR primer pairs (one forward and one reverse SCAR primer) were tested by means of sub groups; low and high root length types and low and high root dry weight types. Among 127 mapping population obtained from DR-14 x Madagascar, only extreme individuals low and high with respect to root length and root dry weight were selected. To ensure that the SCAR primer pairs correctly identified their respective group of individuals. The PCR products were resolved on a 1.5% (w/v) agarose gel. Presence and absence of the SCAR band was visually scored.

Experimental results

IV EXPERIMENTAL RESULTS

In the earlier study (Awati, 2004), a trait-specific (root traits) mapping population comprising of 134 progeny plants developed by crossing S.3334 (BR.8 11/7 X BR.11 9/15) and a local selection L1 valley robusta (*Coffea canephora* (robusta) was molecular characterized by RAPD markers. The major emphasis of the present investigation was to further molecular characterization of this population using additional marker system including microsatellite markers, in which these microsatellite markers were developed. Further aim is to develop locus specific SCAR markers that are linked to root traits and validate these markers in another trait specific (root traits) mapping population. The results obtained from this experiment are presented in this chapter.

4.1 Development of SSR markers from *Coffea robusta* genomic DNA

Microsatellites or SSRs (simple sequence repeats) are short tandem repeats of DNA that are widely dispersed in all eukaryotic genomes. The SSR markers are PCR amplified by using specific primers designed from unique flanking regions. Microsatellites are generally co-dominant and highly polymorphic. This high level of detected polymorphism mainly results from a variable number of repeats, primarily owing to replication slippage, although unequal crossing over might also be involved in the genome (Leevinson and Gutman 1987). These markers are increasingly being used for germplasm diversity, linkage analysis and molecular breeding (Gupta & Varshney, 2000). Despite these advantages lesser extent of microsatellite markers have been reported in coffee (Combes et al, 2000; Rovelli et al 2000), signifying the need for expanding the repertoire of these genetically highly informative markers for efficient management and improvement of coffee germplasm resources. In this study, twenty-three new coffee-specific microsatellite markers were developed from a partial genomic library for use as potential genetic markers.

4.1a Restriction digestion and Linker ligation to digested DNA fragments

DNA isolated from leaves of elite *C. canephora* var. S.3334 was digested with *Rsa*I enzyme to fragment the DNA into approximately 500bp (Plate 3a). The fragmented DNA was ligated with double stranded super SNX linkers, so that this linker provides the primer-binding site for the subsequent PCR steps and also provides the sites for cloning of fragment into the vectors. To ensure the linker ligation has worked, PCR of linker ligation mix was performed and 5µl of PCR product was resolved on 1.2% agarose gel, using 1kb ladder as a standard (Plate 3b).

4.1b Enrichment of microsatellites containing genomic DNA fragments

The enrichment reaction was carried out to enrich the repeats from linker ligated PCR product and removing other unwanted DNA fragments. The biotinylated oligos containing di, tri and tetra repeats were hybridized to the DNA fragments that they most closely match at temperature slightly above or below the oligos melting temperature. This product (DNA + probe mix) was added to the washed streptavidin dyna beads. These beads captured the biotinylated probes hybridized to the DNA under magnetic particle collecting (MPC) unit. Again these beads were washed with 2x & 1x hybridization and washing solutions to remove other DNA fragments. At the end 200µl TE (Tris EDTA) buffer was added vortexed and incubated at 95°C for 5 min. and then captured using MPC unit. The supernatant containing the enriched fragments of microsatellite repeats pool (i.e., "GOLD") was collected. This enriched product was further precipitated with 95% ethanol and resuspended in 25µl of TE buffer (pure "GOLD").

To increase the amount enriched microsatellite repeat fragments (pure GOLD DNA), PCR was performed using pure gold DNA as template to recover the enriched product and the PCR product was electrophoresed on 1.0% agarose gel (Plate 4).

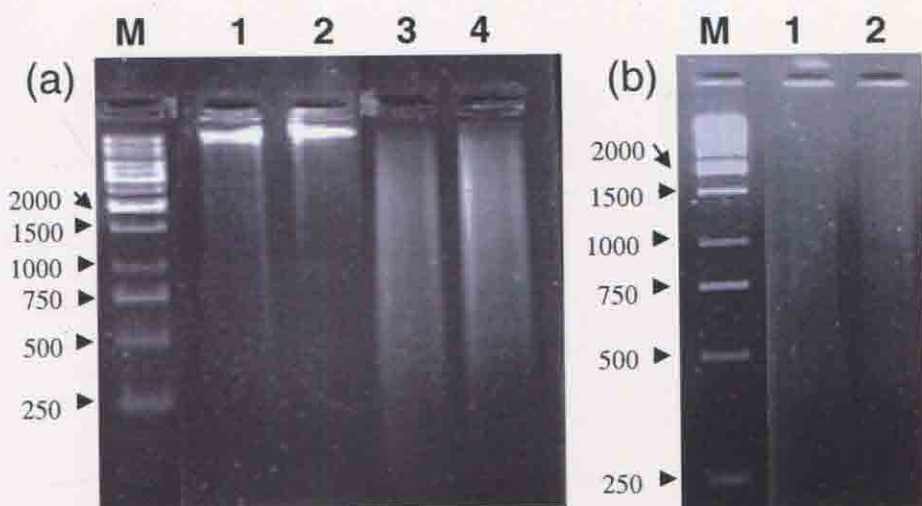


Plate3. Restriction digestion of genomic DNA and Linker ligation to digested DNA fragments. DNA isolated from leaves of elite *C. canephora* var. S.3334 (lane 1 & 2) was digested with *RsaI* enzyme (lane 3 & 4) to fragment the DNA into approximately 500bp (a). The digested DNA fragments were further ligated with double strand super SNX linkers, to provide site for primer binding and cloning of fragments into the vectors. To ensure linker ligation, the linker ligated DNA was PCR amplified with SNX24 forward primers (lane 1 & 2) (b) ; M-1kb ladder.

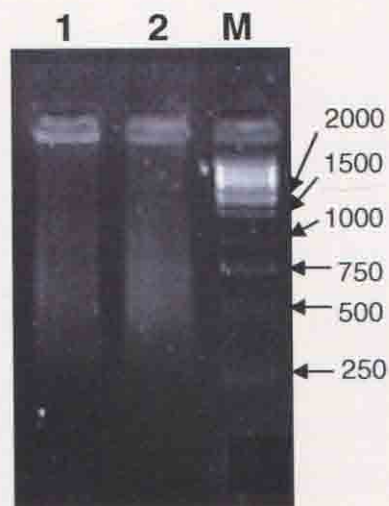


Plate 4. Enrichment for microsatellite containing genomic DNA fragments. In enrichment reaction the unwanted DNA fragments were removed and repeat DNA fragments were captured. To increase the amount of enriched repeat fragments the PCR was performed using the enriched DNA fragment pool as a template with super SNX Forward primer. The super enriched PCR product (lane 1 & 2) was electrophoresed on 1% agarose gel; M-1kb ladder

4.1c Cloning and transformation of plasmid DNA

The amplified enriched recovered microsatellite repeat fragments (pure gold DNA) were cloned into pTZ57R/T vector and transferred into *E. coli*. Based on blue white selection, the presumed recombinant clones were confirmed by cloning PCR reactions, and product was resolved on 1.0% agarose gel (Plate 5).

4.1d Screening and sequencing of recombinant clones

More than five hundred colonies were screened to get the bigger fragment size of 500-1000bp. However one hundred and fifty recombinants with bigger size fragments were selected and further, these clones were PCR amplified for sequencing. These PCR products were purified using PCR purification kit and sequenced with nested M13 forward and reverse primers using ABI prism sequencer. The sequence information revealed that most of the sequences having the average size between the 800-1000bp, however few of having <500bp were identified (Fig. 3).

The sequences were analyzed for the presence of microsatellite regions using the web-based Tandem Repeats finder programme (<http://bioinfo.lifl.fr/mreps/mreps.php>). Out of the 150 expected sequences, only 78 sequences containing the microsatellite repeats and rest of the 72 sequences were identified as false positives (clones without repeats) in the library. Consequently the actual proportion of sequences that contained an SSR motif was further reduced to almost 50%.

The generated sequence information from the library designates the more of di-nucleotide repeats (47%) than the mono (27%), tri (12%) and tetra (14%) nucleotide repeats (Table 2), however no penta and hexa-nucleotide repeats were identified. In di-nucleotide repeats, the GA-based repeat motifs were the most abundant (34%) in the library followed by AG repeats (11%) (Table 3). In contrast, tri-nucleotide and tetra-nucleotide repeats were the least successful in the enrichment library compared to mono and di-nucleotide repeats (Table 4). On the whole, sixty four percent of the repeat motifs (98) identified were less than 15 bp long. The summary of the total number of sequences generated in library is shown in Table 2 and the sequence information of the 150 clones were presented in the Appendix-1

Table 2. Sequences generated from the enrichment library

Library	No. of colonies screened	No. of clones sequenced	Clones without repeats	Nucleotide repeats				Repeats length		Total no. of repeats
				Mono	Di	Tri	Tetra	10-15bp	>15bp	
Enrichment	> 500	150	72	42	72	18	21	98	55	153

More than 500 clones were screened to get the bigger fragment size, however one hundred and fifty bigger fragment clones ranging between 500-1000bs were sequenced with nested M13 forward and reverse primers using ABI prism sequencer. The sequences were analyzed for the repeat motifs regions using the web-based Tandem Repeats finder programme. The results revealed that one hundred and fifty thee repeat motifs were identified, however most of the repeats were very short length of about 10-15bp land and few of them are >15bp long (55). In fact the di-nucleotide repeats were most dominant than mono, tri and tetra-nucleotide repeats.

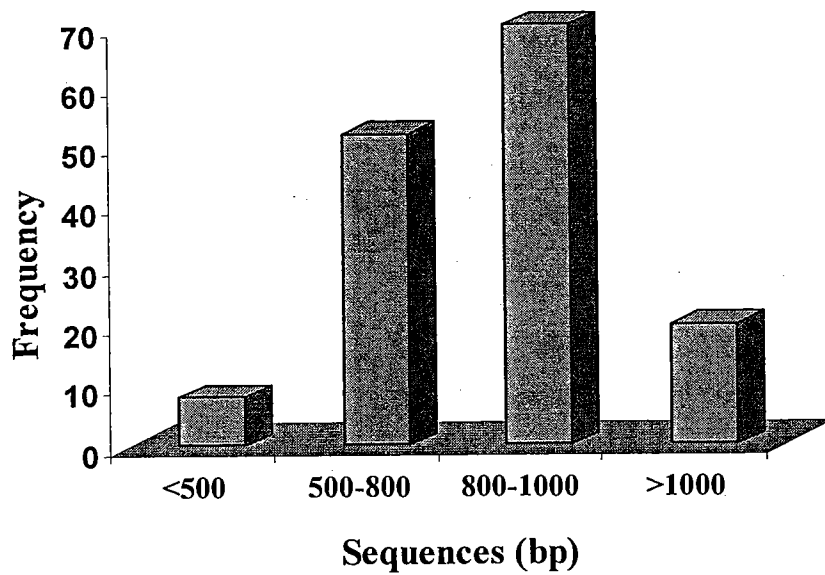


Fig. 3. A graph showing size distribution of clones sequenced from the enrichment library. More than 500 colonies were screened to get the bigger size of the fragments range between 500-1000bp. Finally 150 clones were selected and sequenced. The detailed graphical representation infers the distribution of clone sizes and were classified into four groups based on number of base pairs per sequences.

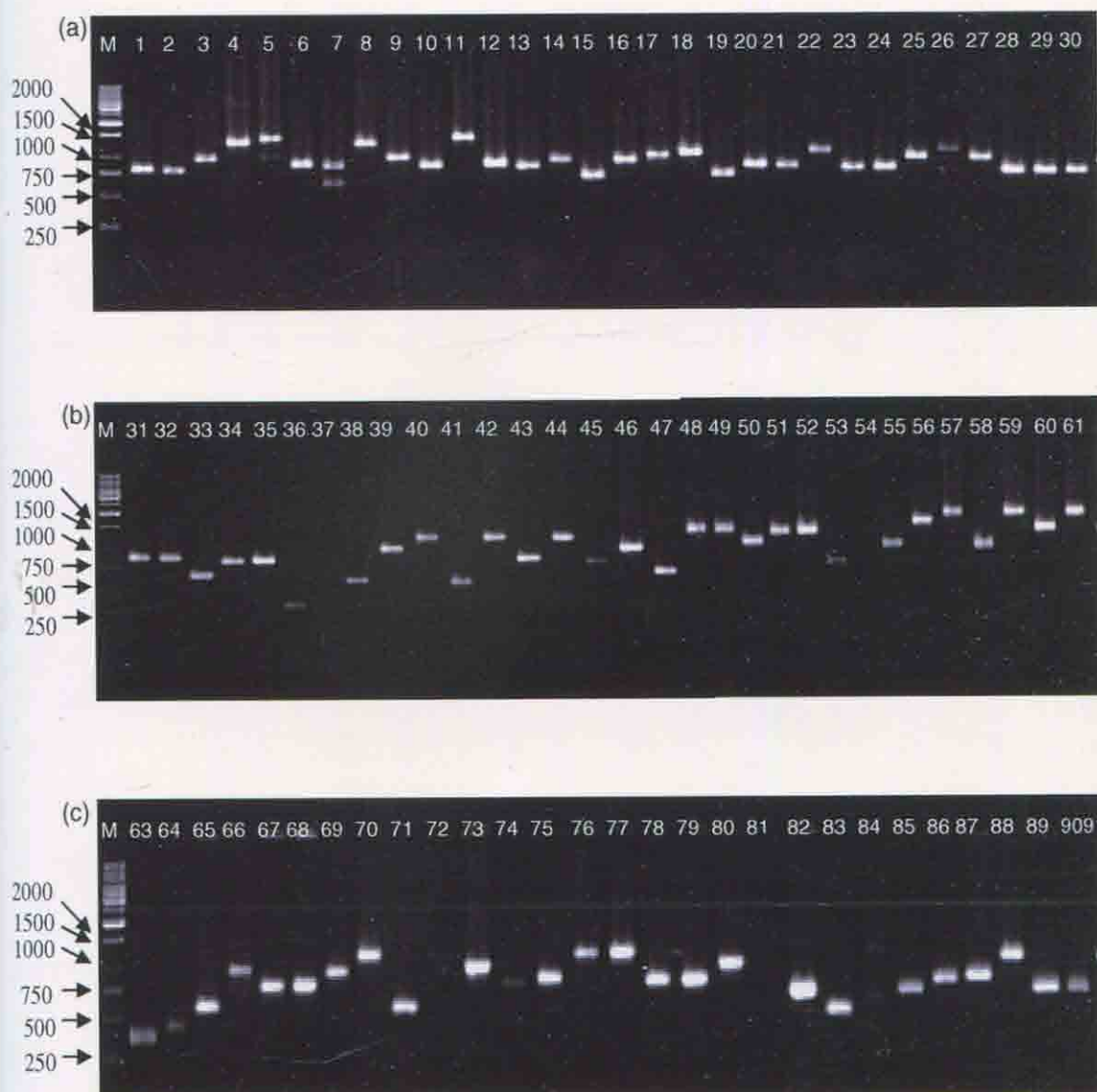


Plate 5. Cloning and transformation of plasmid DNA into T/A cloning vector.

The amplified, enriched, recovered microsatellite repeat fragments (pure gold DNA) were cloned into pTZ57R/T (T/A cloning vector) and transferred into *E. coli*. Based on blue white selection, the recombinant clones were confirmed by colony PCR reactions and product was resolved on 1.0% agarose gel (lane 1-91). Randomly selected clones showed average insert sizes of >500 bp (a, b & c).

4.1e Primer designing and amplification standardization

Of the fifty-five sequences with more than 15bp long, a total of twenty-three primers were designed at the flanking regions of the microsatellites by FastPCR programme (<http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm>) (Table 3 & 4). However thirty-two sequences failed to generate the primers as a result of having bad sequences and as well insufficient flanking regions.

Optimum conditions were established for twenty-three microsatellite loci (Table 5); however three primers could not amplify, thus resulting in 87% of amplification of the primers that were designed. Primers that were not optimized either produced little or no PCR product, or could not amplify reproducible banding patterns. The gradient PCR programme of primer melting temperature $55 \pm 5^{\circ}\text{C}$ (Plate 6) and $60 \pm 5^{\circ}\text{C}$ was followed to amplify the loci. Most of primers generated the sharp bands under the programme of $55 \pm 5^{\circ}\text{C}$ and at $60 \pm 5^{\circ}\text{C}$ the bands were not sharp and often characterized with a smear or spurious bands presumably due to the high stringent annealing temperature. It indicates the high stringent temperature could bring non-specific amplification. The details of the newly developed markers with the repeat motif, primer sequences, standard annealing temperature and product length are summarized in Table 5.

4.1.1 SSR-cross amplification

Patterns of cross-species amplification in plants across genera are beginning to emerge as a source for developing SSRs in lesser-studied species, although only few studies that systematically explore microsatellite transferability beyond closely related genera are available. Rossetto (2001) reviewed cross-species transferability in several plant species. Most of the authors showed the possibility of using SSR primers to amplify heterology with members belonging to the same family (Peakall et al. 1998).

In the present study, the developed microsatellite primer sets from the elite *C. canephora* var. S.3334 (BR 8 11/7 x BR 11 9/15) sequences were used to evaluate the extent to which, these SSR primers amplify homologous loci in nine *Coffea* species (Table 6). In the selected nine species, one species *C. arabica* (Borbon) was self-compatible and rests eight of them were self-incompatible. Out of 23 developed SSR primers, the three primers were not amplified. The genomic DNA of nine species; LIB, REC, CAN, BEN,

Table 3. Distribution and characterization of isolated microsatellite motifs in mono & di-repeat groups

Repeat group	Repeat family	Repeats length		No. of primers designed (>15bp repeats)	No. of primers amplified
		10-15bp	>15bp		
Mononucleotides	A	6	3	-	-
	T	12	3	-	-
	G	9	5	2	2
	C	3	1	-	-
	Total	30	12	2	2
Di-nucleotides	AG	8	9	2	2
	AC	1	0	-	-
	TG	3	4	3	3
	TC	0	1	-	-
	GA	25	7	1	1
	GC	1	0	-	-
	GT	7	0	-	-
	CA	1	1	1	1
	CT	2	2	2	2
	Total	48	24	9	9

Table 4. Distribution and characterization of isolated microsatellite motifs in tri & tetra-repeat groups

Repeat group	Repeat family	Repeats length		No. of primers designed (>15bp repeats)	No. of primers amplified
		10-15bp	>15bp		
Tri nucleotides	AAC	0	2	1	1
	ATC	0	1	1	1
	AGA	0	1	1	1
	AGT	1	0	-	-
	ACA	1	0	-	-
	TCT	1	1	-	-
	TTG	0	1	1	1
	TCT	0	1	-	-
	GAG	1	0	-	-
	GAT	0	2	2	2
	GTT	1	1	1	1
	GTA	0	0	-	-
	GCA	1	0	-	-
	CAT	0	1	1	1
	CTA	0	1	-	-
	Total	7	11	8	8
Tetra nucleotides	AGTG	1	0	-	-
	AGCG	1	0	-	-
	AAAG	1	0	-	-
	AAGG	1	0	-	-
	TTTC	1	0	-	-
	TTCT	1	0	-	-
	GTGA	1	0	-	-
	GTGC	1	0	1	1
	GTGA	1	0	-	-
	GAGT	1	0	-	-
	GAGC	1	0	-	-
	GTGA	1	0	-	-
	CATA	1	0	-	-
	GGTT	0	1	1	1
	GAGT	0	2	-	-
	GTGC	0	1	-	-
	GTGA	0	2	2	2
	TATG	0	1	-	-
	CGTG	0	1	-	-
	Total	13	8	4	4

Table 5. Details of the Microsatellite markers developed in the study

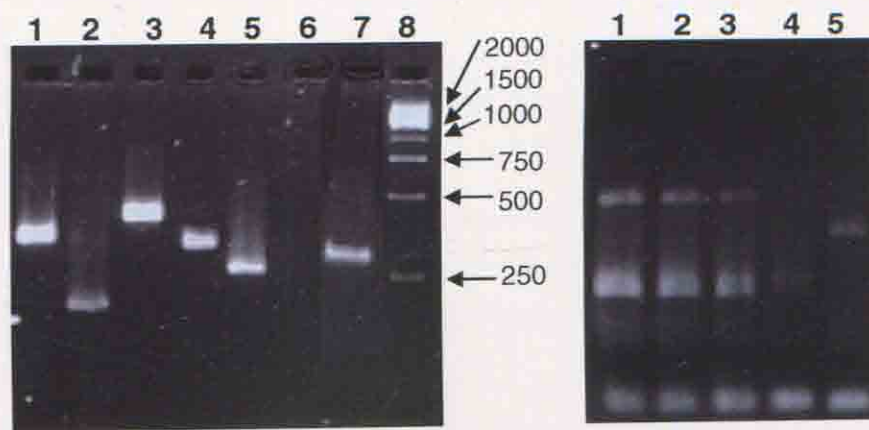
Marker	Repeat motif	Primer sequence (5' --- 3')	Std Ann Temp(⁰ C)	Size range (bp)
CM1	(GTT) ₃ G	F: AGATTGCCAAGAAGTGCAGGGG	54	162-180
		R: TCCGATTGTTTAAGGCCTAGC		
CM2	(AAC) ₃ AA	F: GTTGAGGGAGTTTGGTGCCC	52	260-283
		R: CTAGCTAGCAGAATCACCACG		
CM3	(ATC) ₁₃ AT	F: GACAAGTCCATCGTCCAATCCCGG	52	250-289
		R: ATGCAGGCCTCTGCAGTCG		
CM4	(AG) ₁₁	F: ACCCTCCAATCAAAGTTGCCG	53	210-230
		R: ATTACGATGCTCTCATGCAGC		
CM5	(CAT) ₈	F: TCTCTCTCAAAGCATCTGCG	53	180-216
		R: TTATGGTCAGATGAAGAGGGC		
CM6	(GAT) ₆ GA	F: ACTGCGATCTTCGTCGAACAACG	55	190-270
		R: AGCTAGCAGAATCCATTGCAG		
CM7	(G) ₁₇	F: AGTTTGGGACTGGCGATTCTGC	No amplification	-
		R: CCCCCCAAACCCAGTACG		
CM8	(CT) ₁₆	F: GCTGCTATGCAGCTTGTTTCG	56	262-290
		R: GTCTATACCGATTCCCAAGGC		
CM9	(GAT) ₇	F: CGGTCATCAACAATATCCGCACG	58	207-260
		R: TCTGCTTTTCTAGCAGCTCG		
CM10	(G) ₁₈	F: CCGCGGAAATCCTCCCAACGG	57	240-280
		R: TCCTAACCCACCCACACCCAC		
CM11	(TG) ₁₁	F: GCTGCCAGAAAAATGTTGCAGTG	58	270-338
		R: CTGCCTCGTAAAAGCTTGCGTTG		
CM12	(GGTT) ₅	F: CCTAAGAGTGCAAGGAACTGC	55	203-260
		R: CTGAGTCTGAGAAGCGGCTTTG		
CM13	(CT) ₁₆	F: GCTATGCAGCTTGTTTCGAATCC	59	350-411
		R: CCAGCTATATCAGGAGCAGAACC		
CM14	(TG) ₁₀	F: AGTGATGATGGGGTCACAGG	57	290-373
		R: TTGCATGCAGGCCTTTGCAG		
CM15	(AG) ₁₁	F: AATTGTGCCATTGGTCCGTTTG	65	320-400
		R: GCCAAGCTCTAATACGACTCACT		
CM16	(GA) ₁₂	F: CATTAGGGTGCGTTTGGCGCG	62	280-333
		R: CGAACCCTCAGTCGCCAGTC		
CM17	(TG) ₁₄	F: ACTCAAATAGGCTTGGCGTGCC	60	259-300
		R: TGCAAGTCATGGTGGTGGTGTC		
CM18	(CA) ₁₃	F: AGCTAGCAGAATCCCACTTGC	60	220-261
		R: TGCACCATGCTTCTCGTAGCTG		
CM19	(AGA) ₆	F: AAAGCACCCACGTCGGACAGG	67	300-380
		R: ACGAACTACAGCTGTGTGCAGC		
CM20	(GAGT) ₄	F: AGAGATGTGTGACGAGAGTGG	66	190-233
		R: ACTCACTCACTCGACGTCATC		
CM21	(GAGT) ₅	F: AGTGACGTCAGAGTGAGTGTGC	58	200-261
		R: CGTGTCTACTCACAATCGCACTG		
CM22	(TTG) ₆	F: AATTCCGTGGCGCGAAATCC	No amplification	-
		R: TCACGAACTACAGCTGTGTGCAG		
CM23	(CTA) ₄	F: TACCCCATCACGCGATCTCC	No amplification	-
		R: CACACATCTACAGCAGACAGC		

Note- CM - Coffee Microsatellite

Table 6. Number of SSR primers giving amplification in the nine Coffea species

Sl. No	Species	Mating system
1	<i>C. liberica</i> , LIB	SI
2	<i>C. recemosa</i> , REC	SI
3	<i>C. canephora</i> CV. S. 274, CAN	SI
4	<i>C. bengalensis</i> , BEN	SI
5	<i>C. kapakata</i> , KAP	SI
6	<i>C. abeokutae</i> , ABE	SI
7	<i>C. euginioides</i> EUG	SI
8	<i>C. stenophylla</i> , STE	SI
9	<i>C. arabica</i> CV. Borbon, ARB	SF

Note: SI, Self-incompatible; SF, self-fertile



(a) 55 ± 5°C

(b) 65 ± 5°C

Plate 6. Standardization of annealing temperature of the microsatellite primers. The developed microsatellite primers were initially standardized for the annealing temperature at different gradient temperatures. The gel showing few primers amplification and their standardized annealing temperature at 55 ± 5°C (a) and 65 ± 5°C (b)

KAP, ABE, EUG, STE, ARB were amplified by twenty primers with standardized annealing temperatures as mentioned in the table 5. Out of 20 primers, twelve of them have showed the allelic polymorphism at all nine species. However rest eight primers showed poor amplification with faint band and some of them are showed no amplification, therefore these alleles were considered as non-scorable (very faint) (Table 6a). Few of clearly amplified in all nine species shown in Plate 7. Many of the primers showed heterozygosity at particular locus in many of the species. The proportion of heterozygotic loci in one individual was found to be higher for the self-sterile species KAP (40%). However in the self-fertile species ARB was not showed any heterozygotic loci except in one primer CM 4. Further these markers need to be run on acrylamide gel to confirm their observed amplification in the agarose gel (3%).

4.2 Identification of polymorphic RAPD and microsatellite primers in parental lines of *C. canephora* and screening of mapping population with polymorphic markers RAPD markers

In the present study, the previously (Awati, 2004) identified contrasting parental accessions L1 valley with low root dry weight (6.33g/plant) and S.3334 having high root dry weight (37.40g/plant) were characterized with one hundred and twenty RAPD primers (OPU1 to OPZ20) for parental polymorphism. In polymorphic study, twenty polymorphic primers generated twenty-eight polymorphic markers and these were molecular characterized in existing 134 F1 progenies (L1 valley x S.3334). The obtained molecular binary data from the mapping population was used to study the Mendelian inheritance and trait association study.

In the previous study Awati (2004) screened these parental accessions with two hundred RAPD primers (OPK1 to OPT20). However the 21 primers were identified as polymorphic primers generated 42 polymorphic markers between the parents. The generated polymorphic markers were amplified in 134 F1 progenies. Together from both the studies, out of 320 RAPD primers, 41 polymorphic primers generated seventy polymorphic markers (Table 7). Among them more than 85% of the primers produced single polymorphic locus and 15% of the primers yielded more than three polymorphic loci. Plate 8a to 8c showed the segregating polymorphic loci in the 134 individuals of

Table 6a. Amplification of SSR primers giving detectable amplification on agarose for LIB (L), REC (R), CAN (C), BEN (B), KAP (K), ABE (A), EUG (E), STE (S), ARB (Ar)

	Primers	<i>Coffea</i> species										
		LIB	REC	CAN	BEN	KAP	ABE	EUG	STE	ARB		
1	CM 1	1	1	1	1	1	1	1	0	1		
2	CM 2	1	1	1	1	1	1	1	1	1		
3	CM 3	1	1	1	1	0	0	1	1	1		
4	CM 4	1	1	1	1	1	1	1	1	1		
5	CM 5	1	0	1	0	0	1	0	1	1		
6	CM 6	1	1	1	1	1	1	1	1	1		
7	CM 8	1	1	1	1	1	1	1	1	1		
8	CM 9	1	1	1	1	1	1	1	1	1		
9	CM 10	1	1	0	0	1	0	0	1	1		
10	CM 11	1	1	1	1	1	1	1	1	1		
11	CM 12	1	1	1	1	1	1	1	1	1		
12	CM 13	1	1	1	1	1	1	1	1	1		
13	CM 14	1	1	1	1	1	1	1	1	1		
14	CM 15	1	1	1	1	1	1	1	1	1		
15	CM 16	1	1	1	1	1	1	1	1	1		
16	CM 17	1	1	1	1	1	1	0	0	1		
17	CM 18	1	1	1	0	0	1	1	1	1		
18	CM 19	1	1	1	1	1	0	0	0	0		
19	CM 20	1	1	0	0	1	1	1	1	0		
20	CM 21	1	1	1	1	1	1	1	1	1		

Note:- CM, Coffee Microsatellite; 1= locus is present, 0= locus is absent

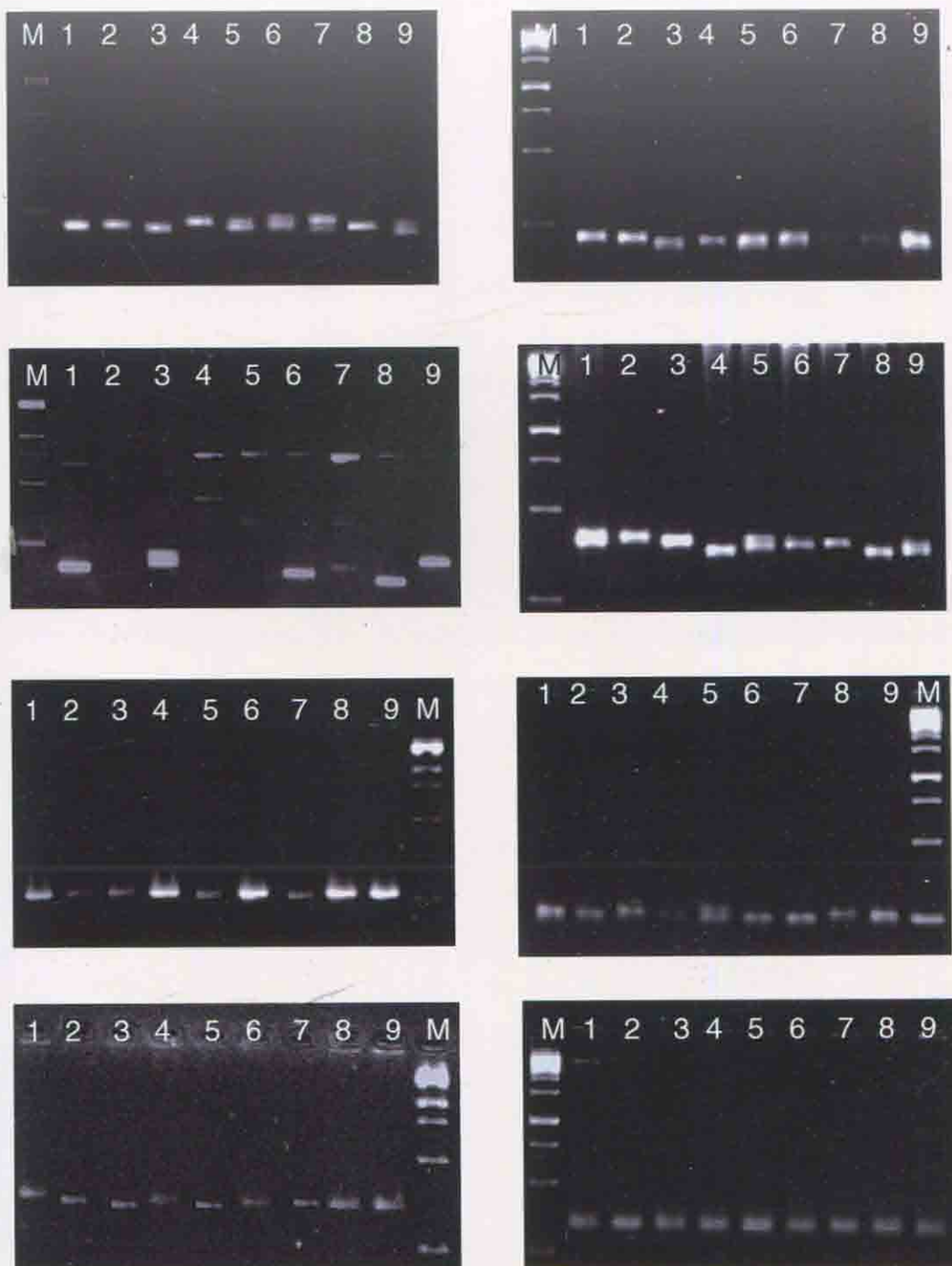


Plate 7. Cross amplification of in house developed SSR markers in nine *Coffea* species.

Representative gels showing the newly developed microsatellite primers such as (1) CM 4, (2) CM 5, (3) CM 8, (4) CM 11, (5) CM 17, (6) CM 13, (7) CM 12 and (8) CM 21

Table 7. Primer screening study in the parental accessions (L1 valley and S.3334)

No. of Primers screened for the parents		Polymorphic primers identified		No. of polymorphic loci identified		No. of marker associated with traits		No. of morphological & physiological traits used in this study
RAPD	SSR	RAPD	SSR	RAPD	SSR	RAPD	SSR	
320	52	41	9	70	9	37	5	29

mapping population. The polymorphic primers and their sequence information from both the studies are furnished in the Table 8.

4.2.1 Microsatellite markers

Apart from the RAPD markers, no other marker system was used in the previous investigation. In the present study microsatellite markers were employed to study the parental polymorphism. Indeed we have used twenty microsatellite primers, developed from one of the parental accession (S.3334) genomic DNA. Together with the 20 in-house developed primers, 32 data base developed primers (<http://www.coffeedna.net/>) were also used in the initial screening to identify the polymorphic primers between the contrasting parents. In the primer screening study, the two in-house developed primers namely CM 11((TG)₁₁) & CM 13 ((CT)₁₆) and seven from the data base (CM180, CM32, CM46, CM20, CM171, CM166 & CM211) showed polymorphism. The generated nine polymorphic loci are used to study the segregation of alleles in the mapping population. The few primers banding pattern and its frequency among the 134 mapping individuals are illustrated in plate 9a and 9b. The polymorphic microsatellite primers and their sequences are used to study the segregation of markers in the mapping population (Table 9).

4.2.2 Study of segregating RAPD and microsatellite markers in the mapping population

Segregation analysis

In dominant marker system (RAPD), marker is present in one parent and absent in the other ($Aa \times aa$ or $aa \times Aa$) the marker will segregate in a 1:1 ratio in the F1 generation. When a marker is present in both parents ($Aa \times Aa$) (co-dominant) the expected segregation ratio for that marker in the progeny is 3:1. We have analyzed our data within the framework of these assumptions. All the seventy RAPD markers in the F1 progenies segregated in ratios that were not consistent with Mendelian inheritance. Out of the seventy RAPD marker loci, forty-two were inherited in a Mendelian manner. However the nine-microsatellite markers were inherited in a non-Mendelian manner (Table 10). Chi-square tests were performed for each of the markers to determine the segregation in

Table 8. Polymorphic RAPD primers and their sequences used to study the segregation of markers in the mapping population

Sl. No	Primer	Sequence	No. of polymorphic bands between parents
1	OPK10	GTGCSSCGTG	2
2	OPK11	AATGCCCCAG	2
3	OPK17	CCCAGCTGTG	4
4	OPK18	CCTAGTCGAG	1
5	OPK19	CACAGGCGGA	1
6	OPK20	GTGTCGCGAG	1
7	OPL1	GGCATGACCT	2
8	OPL12	GGGCGGTACT	2
9	OPL18	ACCACCCACC	2
10	OPL19	GAGTGGTGAC	3
11	OPM11	GTCCACTGTG	2
12	OPM17	TCAGTCCGGG	2
13	OPM2	ACAACGCCTC	3
14	OPM5	GGGAACGTGT	1
15	OPN19	GTCCGTACTG	1
16	OPN6	GAGACGCACA	1
17	OPO1	GGCACGTAAG	1
18	OPO10	TCAGAGCGCC	2
19	OPO16	TCGGCGGTTC	2
20	OPO18	CTCGCTATCC	1
21	OPO4	AAGTCCGCTC	1
22	OPP11	AACGCGTCGG	3
23	OPP2	TCGGCACGCA	1
24	OPP5	CCCCGGTAAC	2
25	OPP9	GTGGTCCGCA	3
26	OPQ20	TCGCCCAGTC	2
27	OPQ5	CCGCGTCTTG	1
28	OPR4	CCCGTAGCAC	2
29	OPS1	CTACTGCGCT	1
30	OPT7	GTCCATGCCA	2
31	OPU1	ACGGACGTCA	2
32	OPV11	CTCGACAGAG	1
33	OPV14	AGATCCCGCC	1
34	OPV2	AGTCACTCCC	1
35	OPV5	TCCGAGAGGG	1
36	OPW17	GTCTTGGGTT	2
37	OPW9	GTGACCGAGT	1
38	OPY20	AGCCGTGGAA	1
39	OPZ10	CCGACAAACC	3
40	OPZ11	CTCAGTCGCA	1
41	OPZ3	CAGCACCGCA	2
	Total		70

Table 9. Polymorphic microsatellite primers and their sequences used to study the segregation of markers in the mapping population

Sl. No	Primer	Sequence	Std Ann Temp (°C)	Size range
1	CM11	F: GCTGCCAGAAAAATGTTGCAGTG	58	270-338
		R: CTGCCTCGTAAAAGCTTGCGTTG		
2	CM13	F: GCTATGCAGCTTGTTTCGCAATCC	59	350-411
		R: CCAGCTATATCAGGAGCAGAACC		
3	CM180	F: CATGTGTAATACATTCAACAGTGA	60	300-350
		R: GCAATAGTGGTTGTTCATCCTT		
4	CM32	F: AACTCTCCATTCCCGCATTC	60	180-200
		R: CTGGGTTTTCTGTGTTCTCG		
5	CM46	F: CAGCTAGTGTGAAGGGAAAC	55	300-350
		R: GTTATCATGGTCTTACACG		
6	CM20	F: CTTGTTTGAGTCTGTGCTG	60	250-300
		R: TTCCCTCCCAATGTCTGTA		
7	CM171	F: TTCCCCCATCTTTTCTTTC	60	250-300
		R: TTGTATACGGCTCGTCAGGT		
8	CM166	F: AAGAGGTGCCTATCACCGTC	60	230-260
		R: CGAGGTATCAAAAAGCACCT		
9	CM211	F: TCATGCCAAATATGAGTGGA	60	300-350
		R: GAGATGGCAAAGGCTGTTC		

Note:- CM, Coffee Microsatellite

Fifty-two microsatellite primers were initially used to study the parental polymorphism (L1 valley and S.3334). The above-mentioned nine primers were found as a polymorphic between the parents. Further these primers were characterized in the 134 F1 progenies of *Coffea canephora* (L1 valley x S.3334) to study the marker segregation and trait association.

Table 10. Chi-square tests (X^2) for RAPD and SSR markers in the Coffea canephora mapping population

Parents	No. of F1 progenies	Marker System	Total Markers	No. of marker loci not following expected ratio	No. of marker loci following expected ratio (1:1 & 3:1)	No. of significant markers		
						$P=0.95$	$P=0.05$	$P=0.001$
L1 valley x S. 3334	134	RAPD (Dominant)	70	28	42	32	3	7
		SSR (Co-dominant)	9	9	-	-	-	-

The X^2 tests were executed for each of the markers to determine if segregation differed from the expected ratios for both dominant (1:1) and co-dominant (3:1) markers. In the dominant marker system the marker is present in one parent and absent in other, the marker will be segregate in a 1:1 ratio. When marker is present in both the parents the expected segregation ratio for that marker in the progeny is 3:1. Within this outline the data was analyzed. The results revealed that, 60% of the RAPD markers segregated in a Mendelian inheritance, however in the case of microsatellite markers expected ratio did not followed the Mendelian fashion.

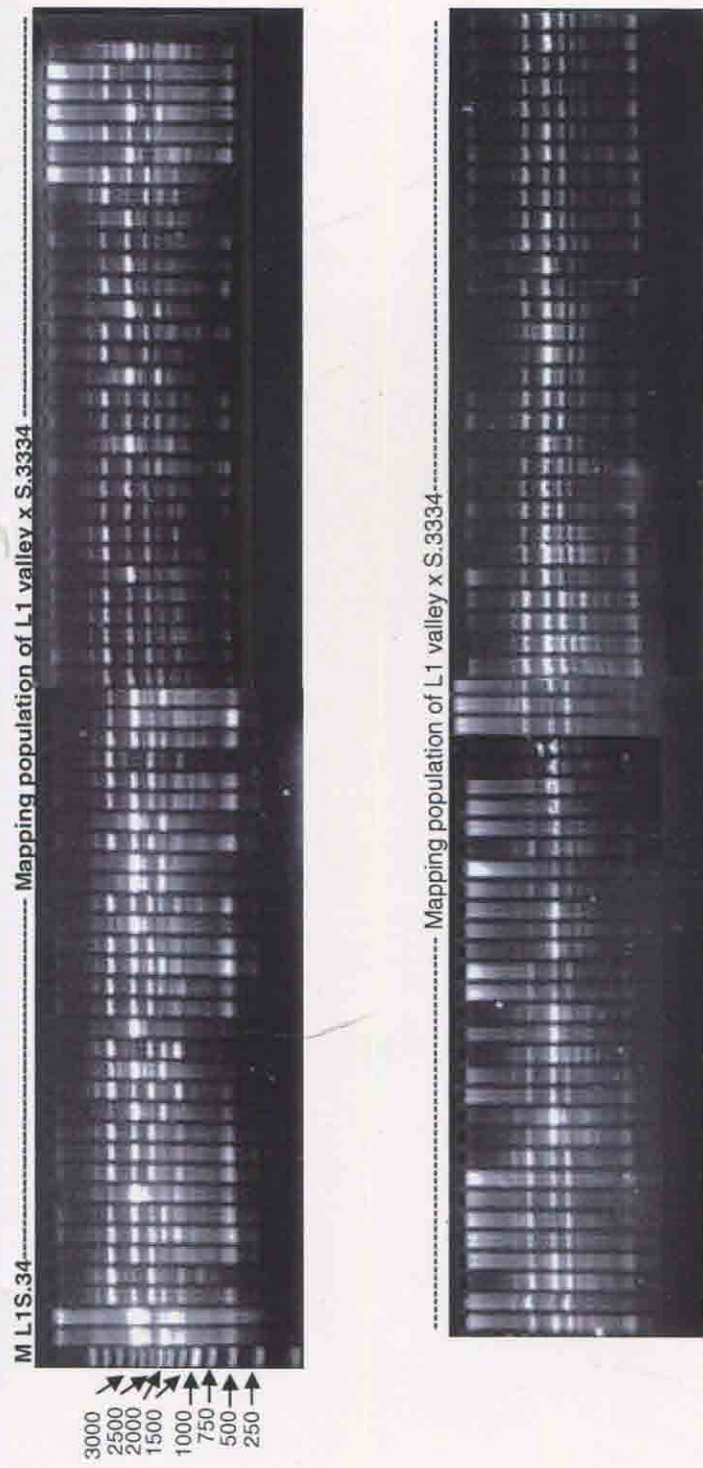


Plate 8a : Segregation of RAPD marker OPZ11 in F1 mapping population of *C. canephora* (L1 valley x S.3334)

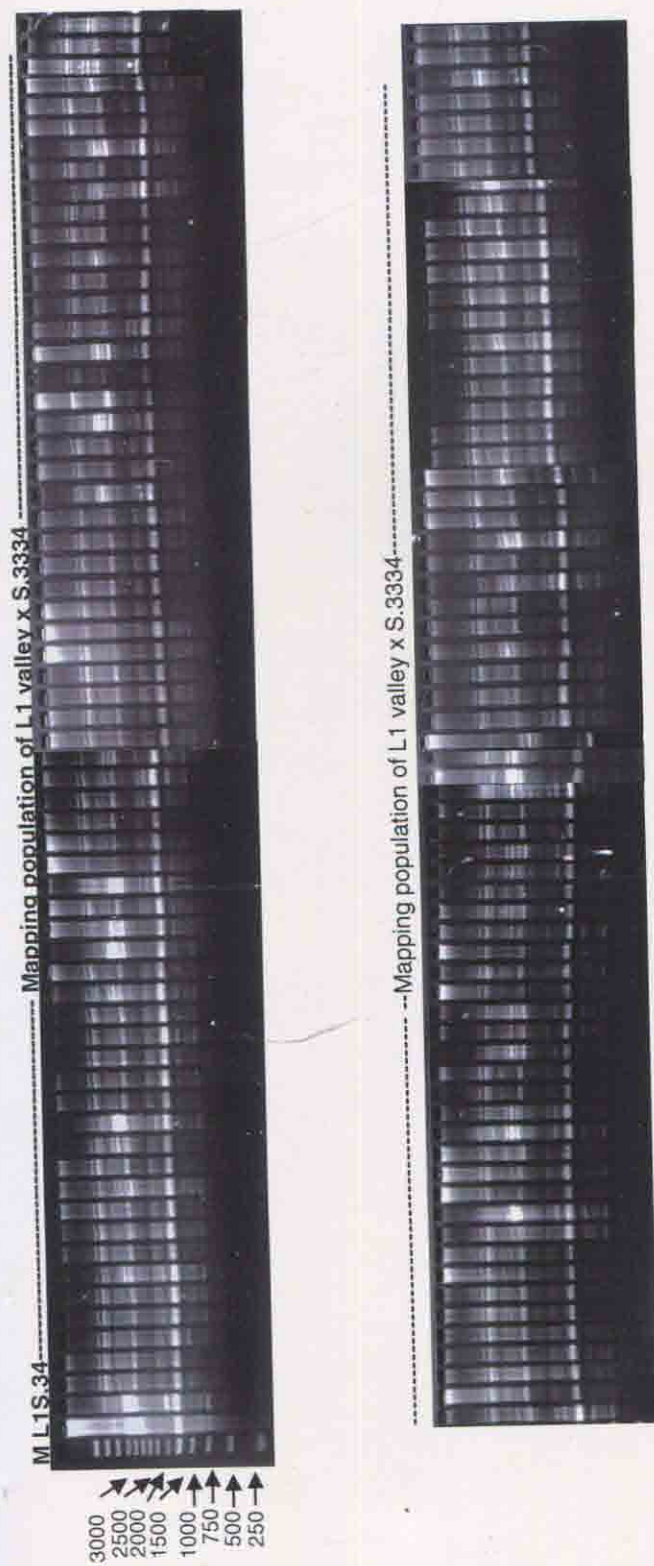


Plate 8b : Segregation of RAPD marker OPZ3 in F1 mapping population of *C. canephora* (L1 valley x S.3334)

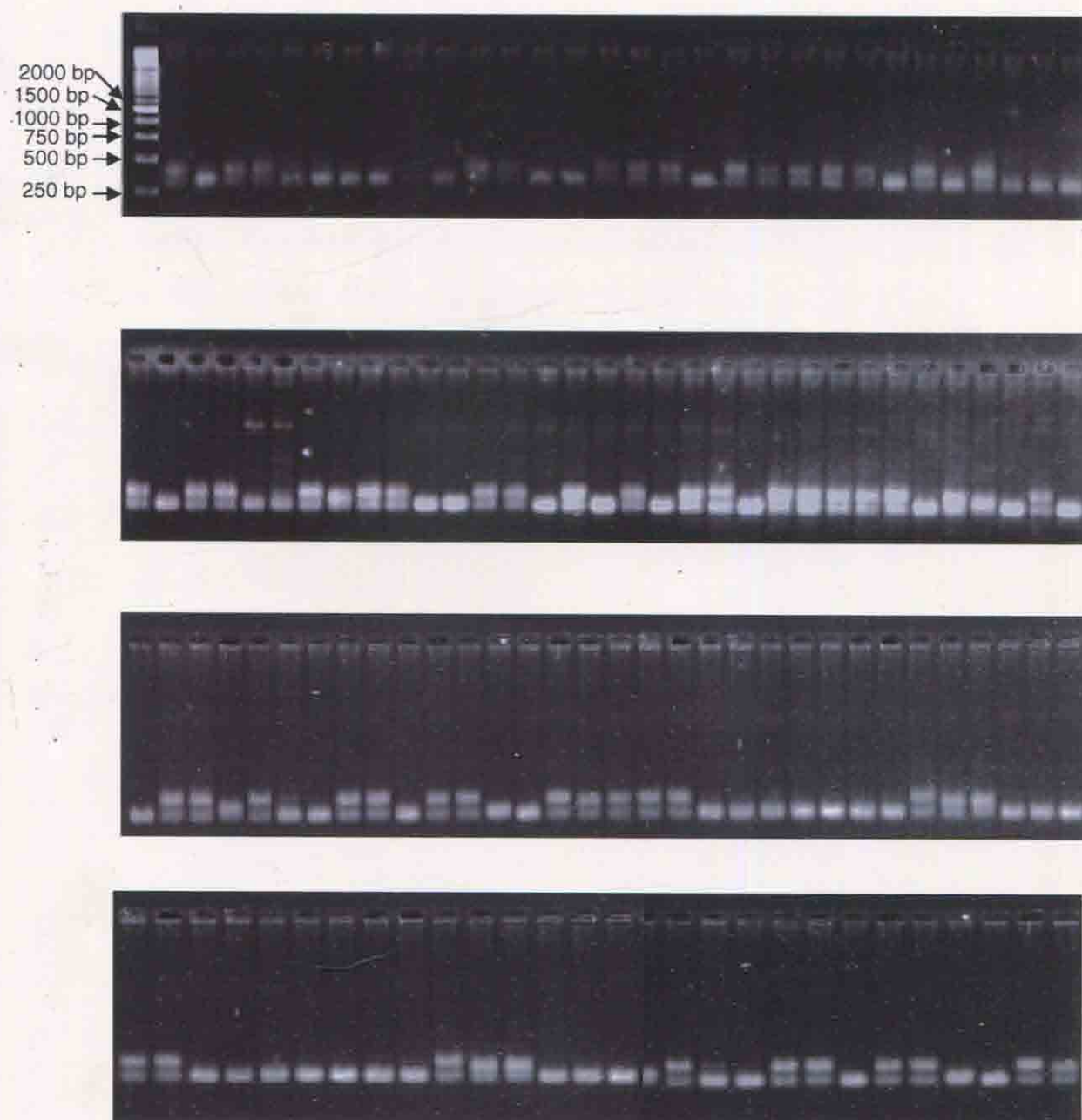


Plate 9. Segregation of microsatellite marker locus **CM180** in F1 mapping population of *Coffea canephora* (L1 valley X S.3334)

expected 1:1 ratios and to find the level of significance of the markers. The markers inherited in a Mendelian inheritance showed significant difference from null hypothesis, for the reason that the calculated X^2 value is not equal or less than the tabulated χ^2 value for 0.95 probability therefore our results gives no reason to accept the null hypothesis. However the markers such as OPL1₁₄₅₀, OPM2₈₀₀ and OPT7₆₀₀ are significant at $P=0.05$ and OPL1₃₀₀₀, OPK17₉₅₀, OPK17₄₀₀, OPR4₆₀₀, OPM2₄₈₀, OPV14₅₅₀ & OPS1₈₅₀ are significant at $P=0.01$ (Table 11). From the chi-square table, it can be assumed that the markers significant at $P=0.05$ are probability that calculated X^2 value would occur by chance only 5%. However at $P=0.01$ probability that calculated X^2 value would be more confident than not occurrence by chance.

4.2.3 Association of different traits with molecular markers

In the previous study (Awati, 2004) the 134 F1 progeny developed by crossing L1 valley and S.3334 was morphologically and physiologically characterized by gravimetric approach. The mean, and range of variation for several traits in the population are presented in Table 12 and the quantitative nature of these traits were shown in Fig 4.

4.2.4 Single marker analysis

In the single marker analysis, simple regression model was examined to study the association between marker loci (independent) and trait score (dependent variable), and also computed the percent phenotypic variance explained by each marker. In this study the SAS package version.8 .0 was adopted to study the marker and trait association.

From the previous study it was observed that, out of 42 RAPD markers, only 21 putative markers were associated with 29 traits. It indicates only 50% of the marker could able to associate with traits. However the objective of present study was, to identify more markers for the traits explaining more variance. Consequently newly identified 28 RAPD and 9 SSR markers were added to the existing 42 RAPD markers data developed by Awati (2004). Using SAS software the data was analyzed at the significance level of 5% ($P=0.05$). From the single marker analysis it was discovered that 37 RAPD and 5 SSR markers were explained their association with morpho-physiological traits. The results of

Table 11. Segregation analysis of RAPD markers in the *Coffea canephora* mapping population

Primers	Fragment size (bp)	Observed F1		X ² value	Probability
P11	1400	78	56	3.610	0.057
	1050	73	61	1.070	0.300
L18	1400	63	71	0.478	0.490
P5	1350	58	76	2.420	0.120
L1	3000	82	52	6.720**	0.010
	1450	81	53	5.850*	0.016
K17	1400	62	72	0.746	0.388
	1100	75	59	1.910	0.167
	950	84	50	8.630**	0.003
	400	84	50	8.630**	0.003
P9	1030	66	68	0.029	0.863
M11	1800	62	72	0.760	0.388
	600	73	61	1.070	0.300
L19	1650	74	60	1.460	0.227
	900	71	63	0.478	0.490
O10	1600	73	61	1.070	0.300
	1400	78	56	3.610	0.057
O1	1350	61	73	1.070	0.300
R4	600	82	52	6.720**	0.010
L12	2000	72	62	0.746	0.388
N19	850	64	70	0.269	0.604
Q20	750	64	70	0.269	0.604
O16	850	64	70	0.269	0.604
	450	70	64	0.269	0.604
K18	1200	67	67	0.000	1.000
M17	1400	69	65	0.199	0.733
	1150	70	64	0.269	0.604
M2	800	55	79	4.300*	0.038
	550	75	59	1.910	0.167
	480	85	49	9.670**	0.002
O18	1100	67	67	0.000	1.000
V14	550	50	84	8.630**	0.003
V2	700	95	39	0.404	0.525
W9	1031	64	70	0.269	0.604
T7	600	55	79	4.300*	0.038
Z3	900	59	75	1.910	0.167
	600	69	65	0.119	0.730
Z10	1350	76	58	2.420	0.120
	1031	63	71	0.478	0.490
	720	62	72	0.746	0.880
K20	350	71	63	0.478	0.490
S1	850	51	83	7.640**	0.006

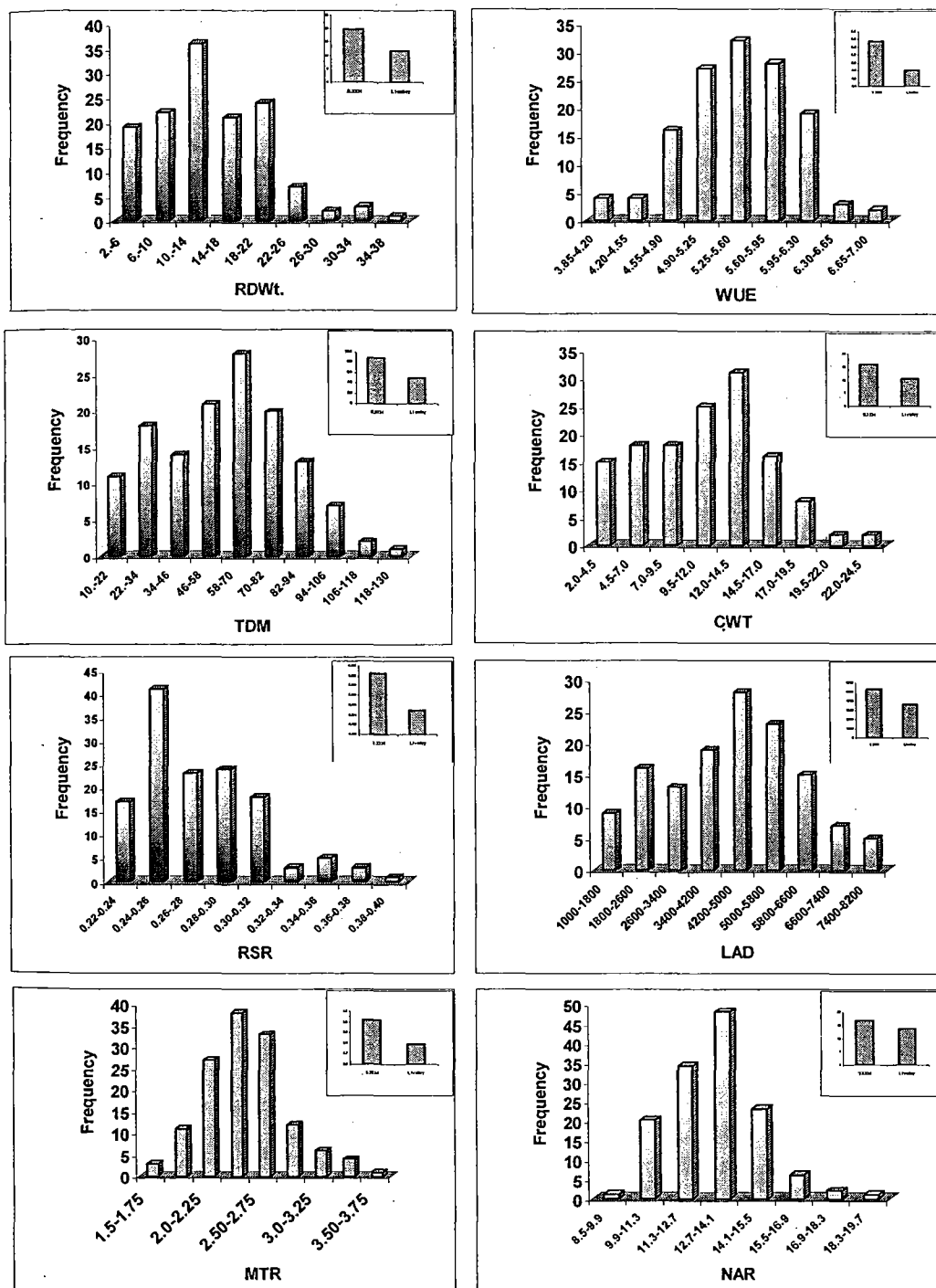
Note: * significant at $P = 0.05$ and **significant at $P = 0.01$

Table 12. Range and mean values of the morpho-physiological parameters in 134 F1 mapping population of *Coffea canephora* (L1 valley x S.3334)

	Sl No.	Parameters	Range	Mean	Std. Deviation
Root traits	1	Root length (cm ²)	41.5 – 83.0	57.08	7.29
	2	No. of secondary roots	2 - 60	26.90	12.61
	3	Root dry weight (g/plant)	2.57 - 34.69	13.77	6.60
	4	Root : Shoot ratio	0.23 - 0.39	0.27	0.03
Morphological traits	5	Plant height (cm ²)	13.5 - 105.3	72.60	15.69
	6	No. of nodes	11.0-42.0	24.08	6.88
	7	No. of leaves	9 - 70	34.02	13.42
	8	Stem girth (mm)	5.88 - 13.65	10.28	1.79
	9	Inter nodal length (cm)	1.9 - 12.5	8.54	2.00
	10	Specific leaf dry weight (mg/dm ²)	1.41 - 2.64	1.80	0.22
	11	Final leaf area (cm ²)	1136.92 - 8190.61	4491.19	1710.17
	12	Specific leaf area (cm)	378.99 - 710.97	562.44	65.34
	13	Total leaf dry weight (g/plant)	5.78 - 48.59	26.02	10.43
	14	Stem dry weight (g/plant)	4.81 - 52.62	24.33	10.40
	15	Shoot dry weight (g/plant)	10.59 - 96.64	50.35	20.63
	16	Total dry matter (g/plant)	11.6 - 121.43	57.93	24.77
Gravimetric traits	17	Leaf area duration (dm ² days)	1065.89 - 7992.77	4372.52	1658.04
	18	Net assimilation rate	8.9 - 19.38	13.07	1.71
	19	Cumulative water transpiration (lt/plant)	2.5 - 23.5	10.81	4.62
	20	Water use efficiency (g/kg)	3.86 - 6.84	5.40	0.58
	21	Mean transpiration rate (ml/dm ² days)	1.5 - 3.52	2.44	0.38
	22	Delta 13c (mill)	19.75 - 26.53	21.80	1.11
Gas exchange parametrs	23	Photosynthesis (Pn). (μmol/m ² /S)	5.45 - 27.27	16.90	3.91
	24	Transpiration rate (E) (μg/m ² /s)	0.71 - 7.47	3.38	1.34
	25	Conductance (gs) (mmol/m ² /s)	0.05 - 1	0.35	0.17
	26	Ci/gs	288.76 - 3484.25	859.30	405.10
	27	Ci	186.5 - 296.67	247.98	24.88
	28	A/gs	24.06 - 93.26	54.42	15.52
	29	A/E	2.63 - 14.07	5.52	1.76

(Awati, 2004)

Fig 4. Frequency distribution of morphological and physiological characters in mapping population (L1 valley x S.3334) of *Coffea canephora*



Legend: RDW = root dry weight, WUE = water use efficiency, TDM = Total dry matter, CWT = cumulative water transpired, RSR = root to shoot ratio, LAD = Leaf area duration, MTR = Mean transpiration and NAR = Net assimilation rate

(Awati, 2004)

newly identified markers associated with their respective traits along with existing markers are presented in this chapter.

4.2.4.1 RAPD markers linked with root traits and other associated physiological traits in *C. canephora*

4.2.4.1a Markers linked to root traits

The four new RAPD markers such as OPS1₈₅₀, OPK11₇₈₀, OPY20₁₂₀₀, and OPZ10₁₃₅₀ were associated with root length. Single marker analysis for this trait revealed significant association ($P=0.05$). These markers explained the total variation in root length of 4.41, 2.95, 3.56 and 5.12 per cent respectively (Table 13).

For number of secondary roots, two newly identified markers such as OPK11₁₀₀₀ (5.51%), & OPV14₅₅₀ (5.82%) were significantly ($P=0.05$) associated, including previously existed marker OPO16₄₅₀ (4.40%). This indicates the existed marker OPO16₄₅₀ is having more strong association for this trait. Similarly for root dry weight only one new marker (OPL1₁₄₀₀ (4.86%)) was associated, however previously identified marker was not existed with new marker. This could be due to newly identified markers are more strong association than previously identified marker. Correspondingly six markers such as OPK11₁₀₀₀ (3.26%), OPP9₁₀₃₀ (3.5%), OPM11₆₀₀ (3.75%), OPL19₉₀₀ (5.61%), OPL19₆₀₀ (4.11%) & OPR4₆₀₀ (3.30%) revealed significant ($P=0.05$) association with the root to shoot ratio (Table 13). In fact marker OPK11₁₀₀₀ was found to be an overlapping marker for number of secondary roots and root to shoot ratio (Table 13). In fact it is also co-associated with other traits like internodal length and stem dry weight. It indicates that traits associated marker locus is same.

4.2.4.1b Markers linked to plant morphological traits

Twenty-nine morphological and physiological traits are considered in the *C. canephora* mapping population (L1 valley X S.3334) for marker loci association in single marker analysis.

In the earlier marker and trait association study, markers did not associate the number of nodes and stem dry weight. From our results the single marker analysis revealed new markers for the number of nodes, stem girth and stem dry weight, and are significantly

Table 13. RAPD markers linked to root traits in the mapping population of *C. canephora* (L1 valley X S.3334)

Trait	Marker	R² (%)	P < 0.05
Root length	OPY20 ₁₂₀₀	2.95	0.0449
	OPZ10 ₁₃₅₀	3.56	0.0286
	OPK11 ₇₈₀	4.41	0.0178
	OPS1 ₈₅₀	6.86	0.0001
No. of Secondary roots	OPO16 ₄₅₀	4.40	0.0179
	OPK11 ₁₀₀₀	5.51	0.0059
	OPV14 ₅₅₀	5.82	0.0042
Root dry weight	OPL1 ₁₄₅₀	4.76	0.0073
Root to shoot ratio	OPK11 ₁₀₀₀	3.26	0.0380
	OPR4 ₆₀₀	3.30	0.0367
	OPP9 ₁₀₃₀	3.50	0.0288
	OPM11 ₆₀₀	3.75	0.0267
	OPL19 ₆₀₀	4.11	0.0223
	OPL19 ₉₀₀	5.61	0.0062

Table 14. RAPD markers linked to morphological traits in the mapping population of *C. canephora* (L1 valley X S.3334)

Trait	Marker	R²(%)	P < 0.05
No. of nodes	OPL18 ₁₁₀₀	7.78	0.0015
Stem girth	OPO18 ₁₁₀₀	3.43	0.0371
Internodal length	OPV11 ₅₀₀	3.24	0.0427
	OPZ10 ₇₂₀	3.58	0.0289
	OPL1 ₁₄₅₀	3.81	0.0240
	OPK11 ₁₀₀₀	4.74	0.0139
	OPP5 ₁₈₀₀	5.82	0.0042
Shoot dry weight	OPP5 ₁₈₀₀	2.71	0.0492
	OPL1 ₁₄₅₀	4.00	0.0163
Stem dry weight	OPK11 ₁₀₀₀	3.44	0.0260
Plant height	OPZ10 ₁₃₅₀	3.03	0.0463
	OPL12 ₂₀₀₀	3.68	0.0306
	OPY20 ₁₂₀₀	4.23	0.0203
	OPU1 ₁₀₃₀	4.23	0.0203
	OPK18 ₁₂₀₀	4.51	0.0145
	OPL1 ₁₄₅₀	5.77	0.0065
No. of leaves	OPY20 ₁₂₀₀	3.99	0.0217
	OPR4 ₇₅₀	5.65	0.0071

($P=0.05$) associated with makers such as OPL18₁₁₀₀ (7.78%), OPO18₁₁₀₀ (3.43%) and OPK11₁₀₀₀ (3.44%) respectively. Similarly five new makers were associated with inter nodal length, among them marker OPP5₁₈₀₀ showed significant association with trait ($P=0.004$), explained the variance of 5.82% (Table 14).

However the plant height was associated with five new markers and one existing marker (OPK18₁₂₀₀). In the newly identified markers, OPL1₁₄₀₀ marker was significantly associated ($P=0.0065$) and explained the total variance of 5.77% than other four associated markers. It signifies the more number of markers in trait association study could identify significant markers, which strongly associated with the traits. The shoot dry weight was not being associated with any marker in the earlier study. In fact our results showed, the markers OPL1₁₄₀₀ (4.0%) and OPP5₁₈₀₀ (2.71%) were found to be associated with shoot dry weight. Together with the existing marker OPR4₇₅₀, a newly identified marker OPY20₁₂₀₀ was associated with number of leaves (Table 14). However existing marker explained more phenotypic variance (5.65%) than the newly identified marker (3.99%). This indicates the adding up of more markers could identify the particular locus controlling traits.

4.2.4.1c Markers linked to plant physiological traits

In the previous study two markers were identified for the specific leaf area (SLA). By adding more markers it was confirmed that, one earlier existed marker OPK18₁₂₀₀ was associated for SLA, however in the present study tow markers were linked to SLW, where they have not identified previously (Awati, 2004). Similarly in the case of leaf area duration (LAD) and net assimilation rate (NAR) the previously associated markers were not existed (Table 15). Nevertheless, two new markers, for instance OPL18₁₀₀₀ (3.12%) and OPP5₁₈₀₀ (7.82%) were associated for the LAD. In fact the marker OPP5₁₈₀₀ is co-associated with shoot dry weight. It is true that the correlation between LAD and shoot dry weight is always positive. This corroborates the present study and we could find that relationship between LAD and shoot dry weight in the mapping population was highly positively correlated ($R^2 = 0.93$) (Awati 2004). Therefore it demonstrated that the trait controlling marker locus OPP5₁₈₀₀ could be same for both the traits. Correspondingly, out of four markers (OPL1₁₄₅₀, OPL19₁₆₅₀ OPM17₁₄₀₀ & OPK20₃₅₀) associated for the NAR;

the marker OPL1₁₄₅₀ is also co-associated with shoot dry weight (Table 15). From this it can be concluded that the trait controlled by the particular marker locus possibly control other related traits.

The two markers OPR4₆₀₀ and OPL12₂₀₀₀ are significantly ($P=0.01$) associated with cumulative water transpired (CWT). The preexisted marker OPR4₆₀₀ showed more variance of about 5.08% than earlier variance of 3.09%. Conversely, no previously associated markers were re-existed for mean transpiration rate (MTR). However three new markers were found to be associated with MTR, wherein significantly associated marker OPZ11₁₅₀₀ showed more variance (8.21%) than other two markers (Table 15). This indicates that saturating with more markers could identify the locus contributing more to the total phenotypic variation.

In the present study we could identify five markers associated for water use efficiency (WUE). Amongst them, marker OPW9₁₀₃₀ was highly significantly associated and explained highest variance of 5.99%. Similarly the ¹³C discrimination ($\Delta^{13}\text{C}$) was associated with six markers; however the marker OPM11₁₈₀₀ was re-existed in the present association study and explained more total variance (4.89%) than other four markers (Table 15).

4.2.4.1d Association of the markers with gas exchange parameters

At single leaf level instantaneous gas exchange parameters were measured and were associated with markers to identify the trait-controlling locus. In this chapter the identified markers for the traits from both present and the previous study was presented.

Of the nine markers associated with photosynthetic rate (P_n), four markers [OPV14₅₅₀ (9.30%), OPZ11₁₅₀₀ (9.23%), OPZ10₁₀₃₀ (7.56%) and OPK19₂₀₀₀ (5.85)] were showed highly significant association ($P=0.001$). In the previous study only three markers were identified for this trait. However preexisted OPK19₂₀₀₀ marker could exist in the present association study and comparatively explained more variance than earlier study (Table 16).

Although the transpiration rate was associated with two markers in earlier study, nevertheless no markers were re-existed in the present study. In fact we could find one new marker (OPP2₁₆₀₀) that could explain the variance of 3.12%. For stomatal

conductance three new markers such as OPR4₇₅₀, OPZ10₁₀₃₀, and OPZ10₇₂₀ showed a significant association, but no previously identified markers were identified in the present study. This indicates the previously associated markers may not be closely associated. Similarly in the case of mesophyll efficiency (Ci/g) the three new markers OPK17₁₁₀₀, OPP9₁₀₃₀ and OPZ10₁₀₃₀ respectively explained the variance of 4.53%, 5.09% and 6.12%. The internal CO₂ (Ci) was associated with two new markers OPP2₁₆₀₀ and OPK20₃₅₀, which explained the variance of 3.13% and 4.32% respectively. However no markers were found earlier for this trait. Correspondingly for the net assimilation rate (A/g) one new marker OPM11₆₀₀ was found to be associated and explained 3.85% variance (Table 16).

4.2.4.1e Association of microsatellite markers to root traits and associated physiological traits

In addition to seventy RAPD markers, nine polymorphic microsatellite markers were used to assess the segregation pattern and to identify markers linked with root traits and other physiological traits in F1 progenies obtained by crossing L1 valley and S.3334 *Coffea canephora* accessions.

The root length and root to shoot ratios are significantly ($P=0.01$) associated with CM13₄₀₀ ($R^2=5.12\%$) and CM211₃₀₀ respectively. Though they are positively correlated, however no overlapping marker was found. Although the specific leaf area (SLA) and specific leaf dry weight (SLW) is inversely related, both are associated with CM166₂₆₀ marker (Table 17). The number of nodes was associated with CM13₄₁₀, CM171₁₉₀, CM166₂₆₀ and CM166₂₄₀ markers. These markers explained the variance between 3.93% and 7.09%. Similarly the stem girth was associated with CM46₁₈₀ and CM 171₂₅₀, the total variance explained by these two markers was 4.13% and 4.54% respectively.

The CM166₁₈₀ and CM211₃₀₀ are significantly ($P=0.01$) associated with transpiration rate (E), explaining the variance between 4.18% and 4.23%. The mesophyll efficiency (Ci/g), intrinsic WUE (A/g) and photosynthetic rate (Pn) are respectively associated with CM13₄₀₀ (4.83%), CM171₂₁₀ (3.45%) and CM171₁₉₀ (3.72%) (Table 17).

However, further analysis with more microsatellite markers is required to refine predictive values for economic traits and positions of QTL that are reliable for actual phenotypic prediction.

Table 15. RAPD markers linked to physiological traits in the mapping population of *C. canephora* (L1 valley X S.3334)

Trait	Marker	R ² (%)	P = 0.05
Specific leaf area (SLA)	OPK18 ₁₂₀₀	4.7	0.0143
Specific leaf weight (SLW)	OPP9 ₁₀₃₀	3.33	0.0356
	OPL19 ₉₀₀	4.66	0.0148
Leaf area duration (LAD)	OPL18 ₁₀₀₀	3.82	0.0222
	OPP5 ₁₈₀₀	7.12	0.0024
Net assimilation rate (NAR)	OPM17 ₁₄₀₀	3.95	0.025
	OPL1 ₁₄₅₀	4.27	0.0149
	OPL19 ₁₆₅₀	4.34	0.0188
	OPK20 ₃₅₀	4.82	0.0132
Cumulative water transpired (CWT)	OPR4 ₆₀₀	5.08	0.0109
	OPL12 ₂₀₀₀	4.74	0.0126
Mean transpiration rate (MTR)	OPM11 ₆₀₀	2.94	0.0467
	OPZ10 ₁₀₃₀	4.38	0.0116
	OPZ11 ₁₅₀₀	8.21	0.006
Water use efficiency (WUE)	OPZ10 ₁₃₅₀	3.02	0.041
	OPV14 ₅₅₀	3.10	0.0326
	OPK19 ₂₀₀₀	4.43	0.0147
	OPK20 ₃₅₀	4.45	0.015
	OPW9 ₁₀₃₀	5.99	0.0056
¹³ C discrimination ($\Delta^{13}\text{C}$)	OPL12 ₁₆₅₀	3.16	0.0456
	OPV2 ₇₀₀	3.16	0.0454
	OPW17 ₅₀₀	3.38	0.0385
	OPW17 ₁₃₀₀	3.64	0.0316
	OPZ10 ₁₃₅₀	4.12	0.0221
	OPM11 ₁₈₀₀	4.89	0.0124

Table 16. RAPD markers linked to gas exchange parameters in the mapping population of *C. canephora* (L1 valley X S.3334)

Trait	Marker	R²(%)	P < 0.05
Photosynthetic rate (Pn)	OPL1 ₁₄₅₀	2.61	0.0429
	OPL12 ₁₆₅₀	3.07	0.0461
	OPZ10 ₇₂₀	3.14	0.0435
	OPO16 ₈₅₀	3.50	0.0353
	OPT7 ₁₂₀₀	3.74	0.0295
	OPK19 ₂₀₀₀	5.85	0.0061
	OPZ10 ₁₀₃₀	7.56	0.0013
	OPZ11 ₁₅₀₀	9.23	0.0005
	OPV14 ₅₅₀	9.30	0.0005
Transpiration rate (E)	OPO10 ₁₄₀₀	3.18	0.0424
Stomatal conductance (gs)	OPR4 ₇₅₀	2.93	0.0486
	OPZ10 ₁₀₃₀	2.96	0.0339
	OPZ10 ₇₂₀	3.26	0.0421
Mesophyll efficiency (Ci/gs)	OPK17 ₁₁₀₀	4.53	0.0163
	OPP9 ₁₀₃₀	5.09	0.0108
	OPZ10 ₁₀₃₀	6.12	0.0051
Internal CO₂ (Ci)	OPO10 ₁₄₀₀	3.13	0.0468
	OPK20 ₃₅₀	4.32	0.0145
Net assimilation (A/gs)	OPM11 ₆₀₀	3.85	0.0270

Table 17. Microsatellite markers linked to root and associated physiological traits in the mapping population of *C. canephora* (L1 valley X S.3334)

	Trait	Markers	R² (%)	P < 0.05
Root associated traits	Root length	CPCM13₄₀₀	5.12	0.0105
	Root to shoot ratio (RSR)	CM211₃₀₀	4.96	0.0105
Plant physiological characters	Specific leaf area (SLA)	CM166₂₆₀	7.19	0.0018
	Specific leaf dry weight (SLW)	CM166₂₆₀	2.83	0.0407
Plant morphological characters	No. of nodes	CM166₂₄₀	3.93	0.0254
		CM171₁₉₀	5.07	0.0109
		CM166₂₆₀	5.07	0.0109
		CPCM13₄₁₀	7.09	0.0025
	Stem girth	CM46₁₈₀	4.13	0.0219
		CM171₂₅₀	4.54	0.0512
Gas exchange parameters at single leaf level	Transpiration rate (E)	CM166₂₄₀	4.18	0.0145
		CM211₃₀₀	4.23	0.0158
	Mesophyll efficiency (Ci/gs)	CPCM13₄₀₀	4.83	0.0111
	Net assimilation rate(A/gs)	CM171₂₁₀	3.45	0.0334
	Photosynthetic rate (Pn)	CM171₁₉₀	3.72	0.0266

4.2.4.1f Over lapping markers

In the present study from the single marker analysis, it was found that out of 42 markers, 25 markers are associated with more than one trait and 17 markers are associated with single trait (Table 18). The $^{13}\Delta C$ is the surrogate method to quantify the water use efficiency and it was demonstrated that there is inverse relationship between these two traits. The marker OPZ10₁₃₅₀ was found to be associated with $^{13}\Delta C$ and WUE. In fact the other traits also associated with this marker and this may perhaps leads to pleiotropic effect. Correspondingly a few positively related traits are associated with same marker such as OPP5₁₈₀₀ (LAD & shoot dry weight), OPZ10₁₀₃₀ (MTR & g_s) and OPK20₃₅₀ (Ci & NAR). The other traits associated with markers independently are presented in the Table 18.

4.3 Development of locus specific Sequence Characterized Amplified Region (SCAR) marker from RAPD markers linked to root trait

The development of rapid and reliable sequence characterized amplified region (SCAR) markers linked to root length and root dry weight is one of the objectives of this study. Based on the single marker analysis, two dominant RAPD markers were identified, viz. OPS1_{850bp} ($R^2 = 9.86\%$, $P = 0.001$) and OPL1_{1051bp} ($R^2 = 4.76\%$, $P = 0.0073$), these markers were statistically linked to low root length and root dry weight parent (L1 valley) respectively, and were selected (Table 10) for the development of SCAR marker.

The specific bands selected were re-amplified by specific primers such as OPS1 and OPL1 between two parents (S.3334 and L1 valley) (Plate 10). Further specific polymorphic band associated for root length and root dry weight was individually excised, purified and cloned into pTZ57R/T vector. Bacterial transformation was carried out and colony PCR was performed to confirm the recombinant clone. Based on blue white selection the positive colonies were selected, which amplified a single fragment whose size corresponded with the cloned segment of the polymorphic band. The recombinant clones were sequenced (Plate 11) and using the sequence information; forward and reverse primers were designed by fast PCR software. The primers (forward/reverse) designed for the root length locus was designated as SCARL1 and for

the root dry weight as SCARD1. The developed primers were initially standardized to arrive at the optimum annealing temperature using plasmid DNA. The standardized annealing temperature for the SCARL1 was 52°C and for SCARD1, it was 59°C (Table 19).

4.4 Validation of SCAR markers in the trait specific mapping population of *Coffea canephora*

Before employing the associated markers in the marker-assisted selection, it is essential to validate their association with the traits. One of the major objectives of this study was to validate the developed SCAR markers for root length and root dry weight in the mapping population. Therefore two parental accessions with contrasting root types were identified crossed and mapping population was developed.

4.4.1 Assessment of genetic variability for root traits in *Coffea canephora* accessions

The objective of this experiment was to identify the contrasting root types among the thirty-five *C. canephora* accessions. Therefore the study of genetic variability in root traits and other associated physiological characteristics are recommended as potential tool for selecting contrasting root types in coffee accessions.

To achieve this objective, two pot culture experiments were conducted. In the first pot culture experiment, the 35 *Coffea canephora* accessions were grown under stress (60% FC) and non-stress (80% FC) conditions. As explained in the material and methods, the accessions were morphologically and physiologically characterized through gravimetric approach (plate 3). A considerable genetic variability in root traits was existed in the 35 accessions at both levels of moisture regime (Table 20). However, despite the data on root traits and other associated morpho-physiological characteristics obtained from first pot experiment, the traits are being resolved to a greater degree of precision, the second pot culture experiment was conducted.

In the second pot culture experiment the 35 *C. canephora* accessions were grown under 80% FC soil moisture regimes. The moisture regime was maintained by gravimetric measurements and to compare the results of the first experiment, plants were characterized for morpho-physiological traits.

Table 18. List of RAPD and SSR markers overlapped with more than one trait

Sl. no	Markers	traits	Sl. no	Markers	traits
1	OPK11 ₁₀₀₀	Internodal length	10	OPR4 ₆₀₀	CWT
		Root to shoot ratio			RSR
		No. of sec. roots	9	OPR4 ₇₅₀	No. of leaves
		Stem dry weight			g _s
13	OPK18 ₁₂₀₀	Specific leaf area			Pn
		Plant height	14	OPV14 ₅₅₀	No. of secon. Roots
4	OPK19 ₂₀₀₀	Photosynthetic rate (Pn)			WUE
		WUE	15	OPY20 ₁₂₀₀	Plant height
21	K20 ₃₅₀	NAR			No. of leaves
		WUE			Root length
		Ci	17	OPZ11 ₁₅₀₀	Pn
3	OPL1 ₁₄₀₀	Plant height			MTR
		Internodal length	18	OPZ10 ₁₃₅₀	¹³ ΔC
		NAR			Plant height
		Shoot dry weight			Root length
		Root dry weight			WUE
		Pn	19	OPZ10 ₁₀₃₀	Ci/gs
11	OPL12 ₂₀₀₀	Plant height			Pn
		CWT			MTR
12	OPL12 ₁₆₅₀	¹³ ΔC			g _s
		pn	20	OPZ10 ₇₂₀	g _s
2	OPL18 ₁₀₀₀	No. of nodes			Pn
		LAD			Internodal length
7	OPL19 ₉₀₀	SLW	22	CPCM13 ₄₅₀	Root length
		RSR			Ci/gs
6	OPM11 ₆₀₀	A/gs	23	CM171 ₃₀₀	Stem girth
		RSR			A/ g _s
		MTR	24	CM166 ₂₆₀	No. of nodes
8	OPO10 ₁₄₀₀	Ci			SLA
		E			E
16	OPP5 ₁₈₀₀	LAD			SLW
		Internodal length	25	CM211 ₃₀₀	No. of nodes
		Shoot dry weight			RSR
5	OPP9 ₁₀₃₀	Ci/gs			E
		SLW			
		RSR			

Table 19. SCAR primers and their sequences used to validate markers in the F1 mapping population of *C. canephora* (DR-14 x Madagascar)

Marker	Trait	Primer sequence (5' --- 3')	Ann. temp (°C)	Size range (bp)
SCARL1	Root length	F: GGGTCCCTCGCGATGCATCTA	52	850
		R: CGCTTGTGTACAAATTAAAGCTTG		
SCARD1	Root dry weight	F: AGTTTAAGAGCTCGGGTACC	59	1400
		R: TGAACATGATTACGCCAGC		

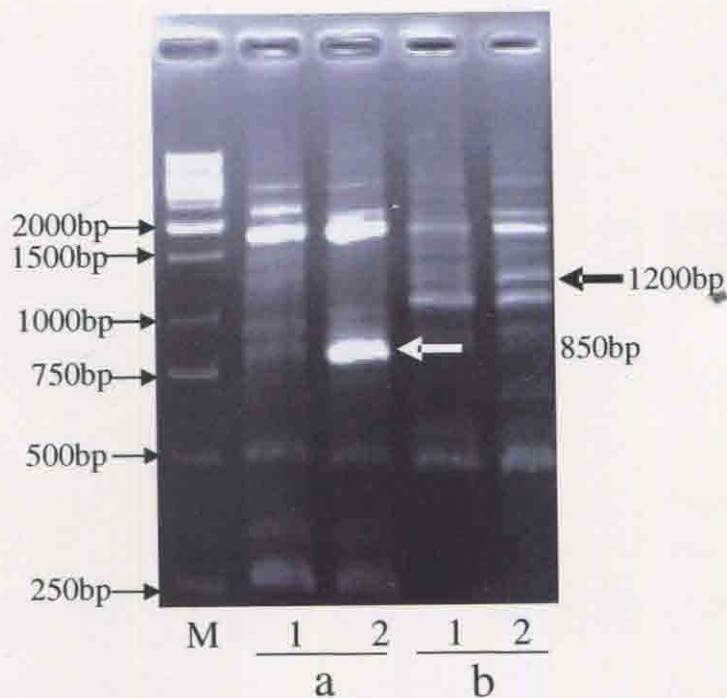


Plate 10. RAPD banding pattern of Operon primers OPS1 and OPL1 amplified between the S.3334 and L1 valley. Based on the single marker analysis, OPS1₈₅₀ and OPL1₁₄₀₀ markers are respectively linked to low root length and low root dry weight parent L1 valley. To reconfirm the required band size, the OPS1 (a) and OPL1 (b) primers were amplified between the parents S.3334 (1) and L1 valley (2) and the product was resolved on agarose gel with 1kb standard marker. Further these bands were excised, purified and cloned into pTZ57R/t vector separately.

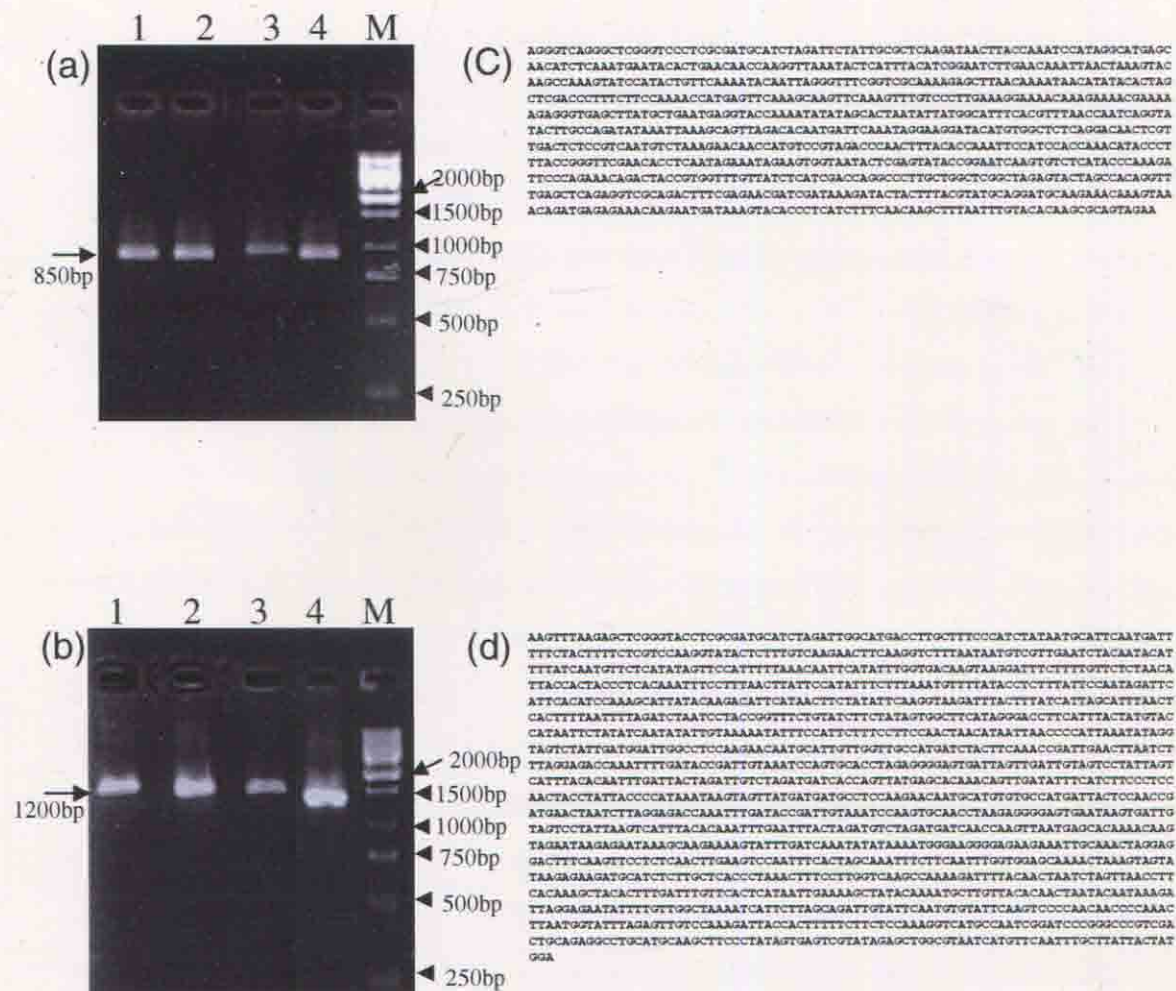


Plate 11. Cloning and sequencing of transformed positive recombinant clones.

The identified dominant RAPD markers OPS1₈₅₀bp and OPL1₁₄₀₀bp bands were excised, cloned into pTZ57R/T (T/A cloning vector) and transferred into *E. coli*. Based on blue white selection, the recombinant clones were confirmed by colony PCR reactions to get a required cloned segment of polymorphic band. The PCR product of four clones (lane 1 to 4) of OPS1₈₅₀ bp marker linked to root length (a) and OPL1₄₀₀ bp marker linked to root dry weight (b) was resolved on 1.0% agarose gel and selected clones showed average insert sizes; M-1Kb ladder. Further, plasmid was isolated from the recombinant clones and sequenced; nucleotide sequences information of OPS1₈₅₀ band (c) and OPL1₁₄₀₀ band (d)

4.4.2 Genotypic variation in a few root traits and their relation with WUE under non-stress and moisture stress conditions

4.4.2a Root dry weight

In the first experiment, root dry weight was recorded on 160 days after transplanting to the pots. In experiment -2 root dry weights was recorded on 210 days after planting to the pots. Significant variation in root dry weight was noticed amongst 35 accessions in two experiments. However the difference in mean root biomass between stress (60% FC) and non-stress conditions (80% FC) was not significant in first pot experiment. But some of the clones like Ivorycost, DR-4, 8 and 20 maintained relatively more root biomass in stress condition (Table 18). It indicates the stress-induced response in the root biomass. Amongst the 35 accessions the clone Madagascar maintained significantly high root dry weight at both the levels of moisture stress (Table 21) and also in the second pot experiment (Table 24). From two pot experiments, the accessions were comparatively maintained the same trend in their root biomass. This further demonstrates the consistency of the accessions in root biomass could be due to low G X E interaction.

A strong positive relationship was observed between the root dry weights under stress v/s non- stress ($r=0.74$) at first pot experiment (Fig 5). Positive correlation in root dry weight between first and second pot experiment was noticed, i.e., root dry weight under stress v/s non stress in experiment-2 ($r=0.54$) and root dry weight under non stress at experiment -1 & experiment - 2 ($r=0.58$) (Fig 6). This indicates intrinsically high root biomass types maintained their high root biomass at both stress and non-stress conditions. This signifies that genotypic expression of root dry weight is independent of stress experienced by the plant (low G x E interaction).

4.4.2b Shoot dry weight

Shoot biomass includes stem and leaf dry weight. A significant difference in the mean shoot dry weight between treatments and among the accessions was noticed at both the levels of moisture regimes (60% & 80% FC). Under moisture stress (60% FC) condition, shoot dry weight was reduced to the extent of 26.60%, but the root biomass was not affected. This indicates maintenance of roots at the cost of shoots under stress condition.

Therefore the shoot dry weight was affected than the root dry weight. The clones DR-10 and Madagascar respectively maintained low and high shoot biomass under both the moisture regimes (Table 21).

However in the second pot experiment, Dwarf C x R and Madagascar maintained low (11.92 g) and high (72.67 g) shoot biomass respectively (Table 24). A positive correlation existed between shoot dry weight in second experiment with 60% FC ($r=0.58$) and 80% FC ($r=0.50$) of first pot experiment (Table 26). In fact, in both the pot culture experiments positive correlation was noticed between shoot dry weight and associated morpho-physiological characteristics such as, TDM, CWT, leaf area, and leaf area duration (Table 26).

4.4.2c Root to shoot ratio (RSR)

There was a significant variation in root to shoot ratio amongst the accessions and between the treatments was noticed. Under non-stress, RSR was ranged from 0.12-0.38, with mean of 0.26 and at stress conditions varied from 0.23-0.45, with mean of 0.33 (Table 20 & 21). However the mean root to shoot ratio under stress condition was increased to an extent of 26.92%. This indicates that root growth is much less affected than the shoot growth under stress condition.

In the second pot experiment (80% FC), the RSR ranged from 0.28-0.61 with a mean of 0.45 (Table 24). It is evident from the two pot experiment that, few genotypes such as Java, Madagascar, DR-1, DR-6, DR-10, DR-18 and DR-19 maintained high RSR at 60% and 80% FC of the first pot experiment have also showed high RSR at 80% FC in the second experiment. The positive relationship ($r=0.52$) between RSR at 60% and 80% FC in the first pot experiment was noticed (Table 26).

Under both stress and non-stress conditions, a positive and significant relationship was observed between root dry weight and RSR (Table 26). This indicates that increase in root dry weight was the contributing factor for increase in RSR. This relationship became stronger when stress & non-stress of the first pot experiment and non-stress of second experiment data were pooled and plotted ($R^2 = 0.52$) (Fig 7).

4.4.2d Total dry matter (TDM)

The mean TDM of 35 accessions under non-stress was 26.40 g and in stress it was 20.66 g. As a result, under stress (60% FC) condition the mean reduction in TDM was 21% (Table 22). The clones, DR-10 and Madagascar maintained low and high total biomass under both non-stress and stress conditions. The same trend was noticed in the second pot experiment also (Table 24).

The contribution of root biomass towards total biomass of the plant was determined by correlation studies. A significant and positive relation was observed between root dry weight and TDM under stress ($r=0.94$) and non-stress ($r=0.92$) in experiment 1 and also in experiment -2 ($r=0.94$), indicating that increase in root biomass in general resulted in increase in total dry matter (Table 26). In general all high biomass types had high root growth.

4.4.3 Genotypic variation in physiological characters under non-stress and moisture stress conditions

4.4.3a Leaf area Duration (LAD)

LAD is the reflection of functional leaf area during the experimental period. There was not much variation in LAD amongst the 35 accessions. However there was considerable decrease in the LAD under stress condition (Table 22). Similarly, in the second pot experiment also variation in LAD among the accessions was marginal (Table 24). Correlations in LAD values under non-stress condition between the two experiments were positive and significant (Table 26). Under stress condition positive and significant relationship was existed between LAD and shoot dry weight ($r=0.63$) & LAD and root dry weight ($r=0.47$) (Table 26).

4.4.3b Cumulative water transpired (CWT)

Cumulative water transpired is the total amount of water transpired by plant during the treatment period. There was a significant variation in CWT amongst the accessions and between the treatments. Under stress it varied from 2.76-11.23 kg/plant and at non-stress it was from 3.68-14.65 kg/plant (Table 20). Correspondingly in the second pot experiment it varied from 5.25 - 35.26 kg/plant. The mean of CWT at non-stress was 9.90

kg/plant, and in stress treatment it was 5.58 kg/plant. The mean CWT under non-stress condition of second pot experiment was 17.0 kg/plant (Table 22 & 25).

In the first pot experiment, under stress the extent of reduction in transpiration was 38.61%. The clone Madagascar showed a CWT of 14.64 kg/plant at non-stress, 11.23 kg/plant at stress and 35.26 kg/plant under non-stress of second pot experiment (Table 22 & 25) respectively having the biomass of 46.09, 42.58 and 108.84 g/plant. This indicates the TDM is strongly associated with transpiration rate. The same trend was noticed in the both experiments.

A highly significant and positive relationship between root dry weight and CWT was noticed under stress ($r=0.61$) and non-stress condition ($r=0.80$) (Fig 8). Similarly in the non-stress condition of the second pot experiment, the correlation between root dry weight and CWT was significant and positive ($r=0.87$) (Fig 9). The CWT showed positive significant with TDM ($r=0.86$) and LAD ($r=0.61$) under stress and non-stress condition in first & also in second experiment (Table 26). However the low TDM under stress could be due to low CWT. Hence it is indicating that conductance and biomass are positively related. Further the increased leaf area duration positively increased the transpiration rate and their by more conductance. When conductance is more internal C_i will be high and therefore the TDM is high.

4.4.3c Water use efficiency (WUE)

Since coffee is predominantly grown as a rainfed crop, improving the root traits alone may prove counter productive. More root biomass would result in faster depletion of soil moisture. For that reason, besides improving root traits it is desirable to enhance the efficiency of water use (WUE). In the present study variation in WUE amongst coffee accessions was computed. Significant genetic variability was noticed among the accessions at both the levels of moisture regimes (stress & non-stress) in the first experiment and even in non-stress of the second experiment. The mean of WUE at 80% FC was 3.0 g/kg & at 60% FC it was 3.77 g/kg (Table 23) and in the second experiment it was 3.17 g/kg (Table 22). The WUE was relatively high in stress (25%) compare to non-stress condition of the first experiment.

In the first experiment accession Ivorycost (S.1589) maintained high WUE at both the levels of moisture stress (4.23 g/kg at 80%FC and 4.93g/kg at 60%FC). In the second experiment, DR-14 (3.85 g/kg), DR-15 (3.87 g/kg) and Ivorycost (S.1589) (3.19 g/kg) maintained high WUE. In contrast, the robusta bold maintained low WUE in both pot experiments (Table 23 & 25). This indicates that some accessions are stable in WUE trait and show low environmental interaction. These kinds of accessions are better for the breeding programme.

There was no correlation between root dry weight and WUE. Thus the negative relationship was observed between CWT and WUE in 80% FC ($r=-0.34$) and 60% FC ($r=-0.49$) of the first experiment and 80% ($r=-0.52$) FC of the second experiment (Table 26). The negative relationship became stronger when the stress data of first experiment and non-stress of the first & second experiment data were pooled and plotted as showed in the Fig 10.

4.4.3d Mean transpiration rate (MTR)

Significant variation in the mean transpiration rate was observed amongst the accessions and at both the levels of moisture regimes. The mean of MTR under stress was 2.11 ml/dm² days and under non-stress it was 3.29 ml/dm² days (Table 23). In second experiment it was 4.41 ml/dm² days (Table 25). Under stress condition the MTR reduced to the extent of 35% over non-stress.

The clone Madagascar has maintained high mean transpiration rate under 80% FC (4.48 ml/dm² days) & 60% FC (3.32 ml/dm² days) of the first experiment and in the second experiment (7.81 ml/dm² days) (Table 23 & 25). The other accessions such as DR-9, 10, 14 and 15 maintained relatively low MTR (1.32-2.24 ml/dm² days) in both the experiments.

In the first pot experiment, there was strong positive and significant relationship between MTR and TDM under both stress and non-stress conditions with an r-value of 0.75 and 0.51 respectively and in the second experiment also ($r= 0.77$) (Table 26).

A more strong negative relationship between MTR and WUE was found under both experiments (Fig 11).

4.4.3e Net assimilation rate (NAR)

NAR is the amount of biomass produced per unit leaf area per unit time. A significant variation among the accessions was noticed under both experiments. In the first experiment, under stress it ranged from 5.0-12.60 mg/dm² days and under non-stress it varied from 5.95-14.09 mg/dm² days (Table 20), where as under second experiment it ranged from 8.69-24.12 mg/dm² days. Our result demonstrates that the in the pot experiment, NAR is more under non-stress condition (9.56 mg/ dm² day) compared to stress condition (7.83 mg/ dm² day). In second experiment mean of NAR was 13.48-mg/dm² days. This corroborates our results that, NAR is significantly correlated with TDM and CWT under stress with r-value of 0.92 & 0.75, and non-stress with an r-value of 0.87 & 0.52 respectively. The same trend was maintained in second experiment (r=0.85 & r=0.83)(Table 26).

4.4.3.1 Identification of contrasting accessions for root traits

With an objective of identifying contrasting parents for root traits, the 35 *Coffea canephora* accessions were characterized for several morpho-physiological traits under stress and non-stress conditions. Using the data from non-stress condition of first and second experiments, standard normalized Z distribution analysis was done for root to shoot ratio and total dry matter (Fig 12). From the four quadrant of Z distribution, contrasting accessions with respect to root to shoot ratio and total dry matter were selected and classified into the following categories.

1. Genotypes with high root to shoot ratio and high total dry matter.
2. Genotypes with low root to shoot ratio and low total dry matter.

The clones Java, Madagascar and Costarica, belongs to category 1, having mean root to shoot ratio of 0.31 and 0.50 in the non-stress condition of first and second experiments respectively. The clones DR-10, DR-14 and DR-15 had low mean RSR of 0.18 and 0.34, in the first and second experiment respectively. This indicates that the trait is genetically controlled and hence is low GxE interaction. Typical root system of high and low root types under first and second pot experiments are shown in Plate 12 and 13. Further, these accessions were characterized for their genetic distance by genetic markers.

Since the root weight is associated with water use, it is desirable to identify accessions with not only the high root biomass but also with high WUE. However there was no relationship between WUE and root biomass under both pot experiments (Table 26).

Although, in the coffee accessions variability in WUE is brought about by differences in stomatal factors there are a few accessions, which showed relatively high WUE and high TDM despite higher mean transpiration rate. To identify such types, the root biomass, TDM and WUE values were transformed and standard normal distribution plot was developed and examined (Fig. 13). Based on normal distribution plot, six accessions were selected as high root biomass with high TDM and moderate WUE and in contrast three accessions having low root biomass with low TDM and WUE were also selected (Table 27). It is well accepted that the increased CWT would certainly decrease the WUE, however in our study we identified some of the accession, which could maintain more TDM with more CWT and WUE. It is an added advantage for the breeding programme. Interestingly the selected high root type clone Madagascar maintained more TDM and WUE under both pot experiments.

4.4.3.2 Assessment of genetic diversity using RAPD markers

After phenotyping, the 35 accessions were genotyped with RAPD markers to study the genetic distances.

The genomic DNA from 35 *C. canephora* accessions were extracted by following the standardized modified protocol of Bhat *et al.* (2002). The 10-mer 200 RAPD primers (OPK1 to OPT20) were tested for genetic analysis of 35 *C. canephora* accessions; however the best 17 primers that rendered polymorphic and reproducible banding pattern were chosen. The primers used and their sequences are listed in the Table 28. Totally 454 bands were produced from the selected 17 primers used in estimating the genetic diversity among 35 robusta accessions. The primers OPQ5 and OPN16 produced maximum number of bands, while the primer OPL2 produced minimum bands (Table 28). Out of 454 bands 143 were polymorphic (31.5%). The selected few gel profiles of 35 accessions are furnished in plate 14a to 14c.

Table 20. Genotypic variation of root traits and physiological characteristics among the 35 *C. canephora* accessions grown under non-moisture stress (80% FC) and moisture stress (60% FC) conditions

Parameters	80% FC			60% FC		
	Range	Mean	CV (%)	Range	Mean	CV (%)
Root dry weight (g/plant)	2.81-11.03	5.47	4.96	2.78-10.52	5.12	4.71
Shoot dry weight (g/plant)	8.92-35.06	20.91	38.84	8.0-32.07	15.54	30.64
Root shoot ratio	0.12-0.38	0.26	0.004	0.23-0.45	0.33	0.005
Total biomass (g/plant)	11.84-46.09	26.40	65.99	10.77-42.58	20.66	55.67
Cumulative water transpired (kg/plant)	3.68-14.65	9.09	8.92	2.76-11.23	5.58	4.65
Water use efficiency (g/kg)	1.58-4.23	3.0	0.51	2.29-4.92	3.72	0.54
Net assimilation rate (mg/dm ² days)	5.95-14.09	9.56	6.06	5.0-12.60	7.83	5.64
Mean transpiration rate (ml/dm ² days)	1.88-5.22	3.29	0.85	1.23-3.40	2.11	0.502

Table 21. Genetic variability in root dry weight, shoot dry weight and root to shoot ratio under non-moisture stress (80% FC) and moisture stress condition (60%FC) – experiment 1.

Sl.No	Accessions	Root dry weight (g/plant)		Shoot dry weight (g/plant)		Root to shoot ratio	
		80% FC	60% FC	80% FC	60% FC	80% FC	60% FC
1	JAVA	9.73	8.83	31.69	23.87	0.31	0.37
2	MADAGASCAR	11.03	10.52	35.06	32.07	0.31	0.33
3	SAIGAN	9.79	8.44	29.42	22.84	0.33	0.37
4	UGANDA880	5.94	6.05	22.89	21.22	0.26	0.29
5	UGANDA1979	3.54	2.99	14.73	11.56	0.24	0.26
6	COSTARICA	8.93	6.05	31.23	17.29	0.29	0.35
7	IVARYCOST	6.56	7.24	26.41	25.07	0.25	0.29
8	UGANDA1977	5.96	6.03	20.98	14.86	0.28	0.41
9	GUATEMALA	5.53	3.06	23.92	12.20	0.23	0.25
10	IVARYCOST3	4.90	3.67	20.62	13.10	0.24	0.28
11	DR-1	6.74	6.16	22.77	19.03	0.30	0.32
12	DR-2	4.26	4.64	18.62	14.58	0.23	0.32
13	DR-3	6.44	4.61	16.92	11.46	0.38	0.40
14	DR-4	2.83	5.33	24.16	19.06	0.12	0.28
15	DR-5	4.77	3.52	18.21	11.57	0.26	0.30
16	DR-6	6.90	5.39	22.52	13.59	0.31	0.40
17	DR-7	4.68	4.22	22.66	14.18	0.21	0.30
18	DR-8	7.35	9.33	25.25	21.38	0.29	0.44
19	DR-9	3.98	2.79	16.97	9.63	0.23	0.29
20	DR-10	2.91	2.78	8.92	8.00	0.33	0.35
21	DR-11	4.42	4.14	17.43	15.31	0.25	0.27
22	DR-12	4.41	4.16	16.05	13.01	0.27	0.32
23	DR-13	7.62	3.53	29.24	12.16	0.26	0.29
24	DR-14	3.50	3.60	17.37	9.98	0.20	0.36
25	DR-15	2.81	3.67	15.81	11.55	0.18	0.32
26	DR-16	4.69	6.85	19.19	16.14	0.24	0.42
27	DR-17	5.59	3.63	21.90	12.60	0.26	0.29
28	DR-18	3.59	3.48	12.06	10.34	0.30	0.34
29	DR-19	4.39	5.55	13.99	12.39	0.31	0.45
30	DR-20	4.83	7.69	23.61	20.50	0.20	0.37
31	SIB-4	6.10	6.60	23.62	22.24	0.26	0.30
32	SIB-7	3.46	2.87	12.25	9.70	0.28	0.30
33	ROBUSTABOLD	3.36	3.45	14.07	10.26	0.24	0.34
34	ROBUSTAYLD	4.22	4.61	17.29	15.49	0.24	0.30
35	DWARF CxR	5.07	3.59	24.11	15.79	0.21	0.23
	Average	5.47	5.12	20.91	15.54	0.26	0.33
CD @ 0.01	Accessions (C)	1.54		2.98		0.08	
	Treatment (T)	NS		0.71		0.02	
	C x T	2.18		4.22		0.11	

Table 22. Genetic variability in total dry matter (TDM), cumulative water transpiration (CWT) and leaf area duration (LAD) under non-moisture stress (80% FC) and moisture stress condition (60% FC) – experiment 1.

Sl.No.	Accessions	TDM (g/plant)		CWT (kg/plant)		LAD (dm ² days)	
		80%FC	60%FC	80%FC	60%FC	80%FC	60%FC
1	JAVA	41.42	32.70	12.63	8.09	2966.41	2776.82
2	MADAGASCAR	46.09	42.58	14.64	11.23	3271.12	3380.41
3	SAIGAN	39.21	31.28	11.54	7.23	3009.82	2759.71
4	UGANDA880	28.83	27.27	8.96	7.34	2302.95	2658.40
5	UGANDA1979	18.27	14.55	4.75	3.08	2434.29	2330.91
6	COSTARICA	40.16	23.34	11.27	5.77	3298.51	2683.57
7	IVARYCOST	32.97	32.31	7.80	6.57	2709.41	2810.18
8	UGANDA1977	26.93	20.89	7.46	4.87	2729.22	2377.31
9	GUATEMALA	29.45	15.26	10.67	3.57	3351.22	2641.12
10	IVARYCOST3	25.52	16.77	7.81	4.16	3518.43	2832.23
11	DR-1	29.51	25.20	10.97	5.87	3111.18	3070.87
12	DR-2	22.88	19.22	7.60	5.46	2177.16	2501.68
13	DR-3	23.36	16.08	7.13	4.19	2829.98	2662.08
14	DR-4	26.98	24.39	7.83	5.75	3255.47	2638.31
15	DR-5	22.98	15.09	6.15	3.38	2371.80	2584.87
16	DR-6	29.42	18.98	9.25	5.42	3374.39	2922.45
17	DR-7	27.34	18.40	7.25	4.12	2952.29	2885.75
18	DR-8	32.60	30.71	9.30	7.37	2511.49	2685.12
19	DR-9	20.95	12.42	6.71	3.06	2139.24	2485.52
20	DR-10	11.84	10.77	3.68	2.76	1961.50	1916.03
21	DR-11	21.84	19.45	5.87	4.68	2700.87	2411.40
22	DR-12	20.45	17.16	12.98	6.09	2899.84	2674.45
23	DR-13	36.85	15.69	13.64	4.62	3169.83	2590.60
24	DR-14	20.87	13.58	8.69	4.77	2855.65	2690.64
25	DR-15	18.62	15.22	9.28	5.28	2533.14	2454.82
26	DR-16	23.89	22.99	8.71	7.38	2424.85	2212.98
27	DR-17	27.49	16.23	8.36	4.29	2410.30	2030.74
28	DR-18	15.65	13.83	5.13	3.92	1978.57	2094.26
29	DR-19	18.38	17.94	8.84	4.67	2311.38	1940.14
30	DR-20	28.43	28.18	12.76	8.78	3201.70	2784.08
31	SIB-4	30.22	28.84	14.26	10.25	2733.90	3606.55
32	SIB-7	15.71	12.56	5.54	3.57	2376.12	2418.48
33	ROBUSTABOLD	17.43	13.71	6.80	4.11	2926.45	2417.44
34	ROBUSTAYLD	21.51	20.10	12.43	8.79	2825.16	2587.46
35	DWARF CxR	29.18	19.38	11.57	4.87	2590.90	3007.06
CD @ 0.01	Average	26.40	20.66	9.09	5.58	2748.99	2614.98
	Accessions (C)	3.84		1.53		422.21	
	Treatment (T)	0.92		0.32		100.93	
	C x T	5.43		2.16		597.09	

Table 23. Genetic variability in water use efficiency (WUE), mean transpiration rate (MTR) and net assimilation rate (NAR) under non-moisture stress (80% FC) and moisture stress condition (60% FC) – experiment 1.

Sl.No.	Accessions	WUE (g/kg)		MTR (ml/dam ² days)		NAR (mg/dm ² days)	
		80%FC	60%FC	80%FC	60%FC	80%FC	60%FC
1	JAVA	3.28	4.04	4.26	2.91	13.96	11.78
2	MADAGASCAR	3.15	3.79	4.48	3.32	14.09	12.60
3	SAIGAN	3.40	4.32	3.83	2.62	13.03	11.34
4	UGANDA880	3.22	3.72	3.89	2.76	12.52	10.26
5	UGANDA1979	3.85	4.72	1.95	1.32	7.51	6.24
6	COSTARICA	3.56	4.05	3.42	2.15	12.17	8.70
7	IVARYCOST	4.23	4.92	2.88	2.34	12.17	11.50
8	UGANDA1977	3.61	4.29	2.73	2.05	9.87	8.79
9	GUATEMALA	2.76	4.27	3.18	1.35	8.79	5.78
10	IVARYCOST3	3.27	4.03	2.22	1.47	7.25	5.92
11	DR-1	2.69	4.29	3.53	1.91	9.49	8.21
12	DR-2	3.01	3.52	3.49	2.18	10.51	7.68
13	DR-3	3.28	3.83	2.52	1.58	8.25	6.04
14	DR-4	3.45	4.24	2.41	2.18	8.29	9.24
15	DR-5	3.74	4.46	2.59	1.31	9.69	5.84
16	DR-6	3.18	3.50	2.74	1.85	8.72	6.49
17	DR-7	3.77	4.46	2.46	1.43	9.26	6.38
18	DR-8	3.51	4.17	3.70	2.74	12.98	11.44
19	DR-9	3.12	4.06	3.14	1.23	9.80	5.00
20	DR-10	3.21	3.90	1.88	1.44	6.03	5.62
21	DR-11	3.72	4.16	2.17	1.94	8.09	8.07
22	DR-12	1.58	2.82	4.48	2.28	7.05	6.42
23	DR-13	2.70	3.40	4.30	1.78	11.63	6.06
24	DR-14	2.48	2.85	3.04	1.77	7.54	5.05
25	DR-15	2.01	2.88	3.66	2.15	7.35	6.20
26	DR-16	2.74	3.11	3.59	3.34	9.85	10.39
27	DR-17	3.29	3.78	3.47	2.11	11.41	7.99
28	DR-18	3.05	3.53	2.59	1.87	7.91	6.60
29	DR-19	2.08	3.84	3.82	2.41	7.95	9.25
30	DR-20	2.23	3.21	3.99	3.15	8.88	10.12
31	SIB-4	2.12	2.81	5.22	2.84	11.05	8.00
32	SIB-7	2.83	3.52	2.33	1.48	6.61	5.19
33	ROBUSTABOLD	2.56	3.33	2.32	1.70	5.95	5.67
34	ROBUSTAYLD	1.73	2.29	4.40	3.40	7.61	7.77
35	DWARF CxR	2.52	3.98	4.47	1.62	11.26	6.44
CD @ 0.01	Average	3.00	3.77	3.29	2.11	9.56	7.83
	Accessions (C)	0.70		0.61		1.66	
	Treatment (T)	0.17		0.15		0.40	
	C x T	0.99		0.86		2.31	

Table 24. Genetic variability in root dry weight (RDW), shoot dry weight (SDW), root to shoot ratio (RSR), total dry matter (TDM) and cumulative water transpiration (CWT) under non-moisture stress (80% FC) – pot experiment 2.

Sl.No.	Accessions	SDW (g/plant)	RDW (g/plant)	RSR	TDM (g/plant)	LAD (dm ² days)
1	JAVA	46.64	25.10	0.54	71.74	4605.55
2	MADAGASCAR	72.67	36.17	0.50	108.84	4512.01
3	SAIGAN	49.08	18.92	0.39	68.00	4649.36
4	UGANDA880	41.46	14.97	0.36	56.42	4283.27
5	UGANDA1979	32.69	16.08	0.49	48.77	4442.13
6	COSTARICA	36.77	18.47	0.50	55.23	3959.73
7	IVARYCOST	59.64	27.14	0.45	86.77	4609.06
8	UGANDA1977	28.78	14.20	0.49	42.98	3382.83
9	GUATEMALA	44.16	23.01	0.52	67.17	4006.83
10	IVARYCOST3	28.95	14.07	0.49	43.02	4092.19
11	DR-1	43.87	20.93	0.48	64.80	3796.03
12	DR-2	25.93	15.69	0.60	41.62	3397.89
13	DR-3	42.09	11.90	0.28	53.99	4959.65
14	DR-4	36.12	15.97	0.44	52.09	4150.11
15	DR-5	35.23	20.05	0.57	55.29	3965.37
16	DR-6	25.86	13.22	0.51	39.08	3875.60
17	DR-7	26.61	11.26	0.42	37.87	3395.23
18	DR-8	37.76	16.61	0.44	54.36	3762.15
19	DR-9	23.97	7.76	0.32	31.74	2969.65
20	DR-10	30.08	11.65	0.39	41.73	3902.08
21	DR-11	23.28	8.54	0.37	31.82	3145.48
22	DR-12	28.91	15.00	0.52	43.91	2692.18
23	DR-13	38.94	16.64	0.43	55.58	3386.77
24	DR-14	32.94	9.46	0.29	42.40	3277.36
25	DR-15	23.86	14.57	0.61	38.43	4420.18
26	DR-16	41.20	20.49	0.50	61.69	4044.46
27	DR-17	46.14	18.00	0.39	64.15	4823.01
28	DR-18	40.93	17.37	0.42	58.30	4231.02
29	DR-19	20.68	9.23	0.45	29.92	3199.78
30	DR-20	22.13	8.62	0.39	30.75	3116.87
31	SIB-4	47.22	18.98	0.40	66.20	3691.90
32	SIB-7	20.33	10.84	0.53	31.17	2734.04
33	ROBUSTABOLD	51.30	18.68	0.36	69.98	4509.21
34	ROBUSTAYLD	32.16	17.50	0.54	49.66	3814.40
35	DWARF CxR	11.92	5.92	0.50	17.84	1369.25
	Average	35.72	16.08	0.45	51.81	3804.93
	CD @ 0.05	3.81	4.66	0.12	6.82	728.48

Table 25. Genetic variability in water use efficiency (WUE), leaf area duration (LAD,)mean transpiration rate (MTR) and net assimilation rate (NAR) under non-moisture stress (80% FC) – experiment 2.

Sl. No.	Accessions	CWT (kg/plant)	WUE (g/kg)	MTR (ml/dm ² days)	NAR (mg/dm ² days)
1	JAVA	25.13	2.85	5.46	15.58
2	MADAGASCAR	35.26	3.09	7.81	24.12
3	SAIGAN	21.94	3.10	4.72	14.62
4	UGANDA880	19.91	2.83	4.65	13.17
5	UGANDA1979	15.00	3.25	3.38	10.98
6	COSTARICA	19.40	2.85	4.90	13.95
7	IVARYCOST	27.17	3.19	5.90	18.83
8	UGANDA1977	12.93	3.32	3.82	12.70
9	GUATEMALA	23.01	2.92	5.74	16.76
10	IVARYCOST3	14.63	2.94	3.57	10.51
11	DR-1	21.74	2.98	5.73	17.07
12	DR-2	11.70	3.56	3.44	12.25
13	DR-3	17.84	3.03	3.60	10.88
14	DR-4	15.46	3.37	3.73	12.55
15	DR-5	14.77	3.74	3.72	13.94
16	DR-6	10.89	3.59	2.81	10.08
17	DR-7	12.80	2.96	3.77	11.15
18	DR-8	14.73	3.69	3.92	14.45
19	DR-9	10.06	3.15	3.39	10.69
20	DR-10	14.85	2.81	3.81	10.69
21	DR-11	10.91	2.92	3.47	10.12
22	DR-12	14.44	3.04	5.36	16.31
23	DR-13	18.03	3.08	5.32	16.41
24	DR-14	11.01	3.85	3.36	12.94
25	DR-15	9.92	3.87	2.24	8.69
26	DR-16	23.10	2.67	5.71	15.25
27	DR-17	18.53	3.46	3.84	13.30
28	DR-18	18.04	3.23	4.26	13.78
29	DR-19	8.14	3.68	2.54	9.35
30	DR-20	10.89	2.82	3.49	9.87
31	SIB-4	28.49	2.32	7.72	17.93
32	SIB-7	11.37	2.74	4.16	11.40
33	ROBUSTABOLD	28.31	2.47	6.28	15.52
34	ROBUSTAYLD	19.27	2.58	5.05	13.02
35	DWARF CxR	5.25	3.40	3.83	13.03
	Average	17.00	3.12	4.41	13.48
	CD @ 0.05	2.36	0.36	0.96	3.02

Table 26. Correlation matrix of root traits and physiological characteristics among the 35 *C. canephora* accessions grown under non-moisture stress (80% FC), moisture stress (60% FC) and experiment 2 at 80% FC conditions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1 SDW S	1.00																													
2 RDW S	0.91	1.00																												
3 RSR S	0.08	0.48	1.00																											
4 TDM S	0.99	0.95	0.20	1.00																										
5 CWT S	0.85	0.83	0.21	0.86	1.00																									
6 WUE S	0.17	0.10	-0.15	0.16	-0.34	1.00																								
7 LA S	0.68	0.49	-0.23	0.64	0.49	0.32	1.00																							
8 LAD S	0.63	0.47	-0.20	0.60	0.61	-0.03	0.79	1.00																						
9 NAR S	0.89	0.93	0.36	0.92	0.75	0.20	0.38	0.24	1.00																					
10 MTR S	0.72	0.78	0.38	0.75	0.91	-0.41	0.18	0.25	0.80	1.00																				
11 SDW C	0.79	0.70	-0.02	0.78	0.60	0.25	0.56	0.63	0.64	0.43	1.00																			
12 RDW C	0.71	0.74	0.25	0.74	0.55	0.23	0.51	0.52	0.63	0.41	0.86	1.00																		
13 RSR C	0.07	0.27	0.52	0.13	0.06	0.08	0.10	-0.05	0.16	0.06	0.02	0.52	1.00																	
14 TDM C	0.79	0.73	0.05	0.79	0.60	0.25	0.56	0.63	0.66	0.44	0.99	0.92	0.15	1.00																
15 CWT C	0.60	0.55	0.03	0.60	0.75	-0.38	0.40	0.65	0.42	0.61	0.70	0.61	0.02	0.70	1.00															
16 WUE C	0.17	0.13	-0.08	0.16	-0.26	0.84	0.23	-0.06	0.21	-0.30	0.25	0.25	0.11	0.26	-0.49	1.00														
17 LA C	0.55	0.45	-0.08	0.53	0.30	0.42	0.50	0.57	0.38	0.11	0.74	0.62	0.03	0.73	0.48	0.28	1.00													
18 LAD C	0.35	0.30	-0.04	0.35	0.31	0.03	0.17	0.59	0.15	0.12	0.61	0.46	-0.11	0.59	0.56	-0.03	0.82	1.00												
19 NAR C	0.76	0.71	0.07	0.76	0.56	0.29	0.60	0.44	0.72	0.47	0.85	0.83	0.19	0.87	0.52	0.34	0.40	0.13	1.00											
20 MTR C	0.52	0.49	0.06	0.52	0.73	-0.47	0.38	0.47	0.43	0.67	0.50	0.46	0.04	0.51	0.89	-0.58	0.13	0.14	0.55	1.00										
21 SDW 2	0.58	0.52	0.05	0.58	0.46	0.15	0.33	0.31	0.50	0.36	0.50	0.58	0.29	0.53	0.29	0.24	0.35	0.24	0.49	0.18	1.00									
22 RDW 2	0.62	0.54	-0.01	0.61	0.50	0.15	0.36	0.33	0.54	0.41	0.55	0.58	0.18	0.57	0.37	0.19	0.42	0.28	0.51	0.26	0.88	1.00								
23 RSR 2	0.08	0.05	-0.12	0.08	0.11	-0.04	0.06	0.06	0.07	0.12	0.10	0.02	-0.19	0.08	0.17	-0.12	0.12	0.07	0.05	0.17	-0.14	0.33	1.00							
24 TDM 2	0.61	0.54	0.03	0.60	0.48	0.15	0.35	0.33	0.53	0.39	0.53	0.60	0.26	0.56	0.32	0.23	0.38	0.26	0.51	0.21	0.99	0.95	0.02	1.00						
25 CWT 2	0.58	0.50	-0.01	0.57	0.53	0.02	0.37	0.37	0.47	0.43	0.47	0.54	0.25	0.50	0.37	0.10	0.37	0.28	0.42	0.26	0.94	0.88	-0.04	0.95	1.00					
26 WUE 2	-0.18	-0.08	0.17	-0.15	-0.29	0.19	-0.19	-0.21	-0.07	-0.23	-0.06	-0.09	-0.11	-0.07	-0.20	0.14	-0.21	-0.19	0.04	-0.11	-0.26	-0.18	0.17	-0.24	-0.52	1.00				
27 LA 2	0.23	0.27	0.17	0.24	0.09	0.27	-0.10	-0.10	0.32	0.14	0.16	0.25	0.19	0.19	-0.18	0.42	0.07	0.06	0.19	-0.25	0.66	0.57	-0.07	0.65	0.55	0.03	1.00			
28 LAD 2	0.24	0.27	0.18	0.25	0.14	0.18	-0.11	-0.07	0.31	0.18	0.17	0.29	0.24	0.21	-0.11	0.36	0.09	0.12	0.18	-0.20	0.71	0.61	-0.08	0.70	0.63	-0.07	0.91	1.00		
29 NAR 2	0.62	0.50	-0.10	0.60	0.54	0.04	0.55	0.51	0.44	0.38	0.58	0.59	0.18	0.60	0.54	0.01	0.44	0.28	0.55	0.46	0.82	0.84	0.10	0.85	0.83	-0.27	0.19	0.22	1.00	
30 MTR 2	0.56	0.43	-0.13	0.53	0.58	-0.12	0.51	0.52	0.36	0.42	0.46	0.48	0.17	0.48	0.54	-0.12	0.40	0.29	0.40	0.46	0.76	0.74	0.02	0.77	0.89	-0.63	0.14	0.22	0.91	1.00

Note: - The correlation values of > 0.325 are significant at 5% and values of > 0.418 are significant at 1%

Legend

SDW - Shoot dry weight, RDW - Root dry weight, RSR - Root shoot ratio, TDM - Total dry matter,
CWT - Cumulative water transpiration, WUE - water use efficiency, LA - Leaf area, LAD - Leaf area duration,
NAR - Net assimilation rate, MTR - Mean transpiration rate,
_S - under stress condition (60% FC), _C - under control condition (80% FC), _2 - Experiment 2 (80% FC)

Table 27. Classification of *C. canephora* accessions based on the root dry weight (RDW), total dry matter (TDM) and WUE under stress and non-stress conditions

No.	High root biomass type accessions	Stress (60% FC) - Expt-I				Non-stress (80% FC) – Expt-I				Non-stress (80% FC) – Expt-II			
		RDW	TDM	CWT	WUE	RDW	TDM	CWT	WUE	RDW	TDM	CWT	WUE
1	JAVA	8.83	32.70	8.09	4.04	9.73	41.42	12.63	3.28	25.10	71.74	25.13	2.85
2	MADAGASCAR	10.52	42.58	11.23	3.79	11.03	46.09	14.64	3.15	36.17	108.84	35.26	3.09
3	SAIGAN	8.44	31.28	7.23	4.32	9.79	39.21	11.54	3.40	18.92	68.00	21.94	3.10
6	COSTARICA	6.05	23.34	5.77	4.05	8.93	40.16	11.27	3.56	18.47	55.23	19.40	2.85
7	IVARYCOST	7.24	32.31	6.57	4.92	6.56	32.97	7.80	4.23	27.14	86.77	27.17	3.19
18	DR-8	9.33	30.71	7.37	4.17	7.35	32.60	9.30	3.51	16.61	54.36	14.73	3.69
	Low root biomass types	RDW	TDM	CWT	WUE	RDW	TDM	CWT	WUE	RDW	TDM	CWT	WUE
24	DR-14	3.60	13.58	4.77	2.85	4.17	21.54	8.69	2.48	9.46	42.40	11.01	3.85
25	DR-15	3.67	15.22	5.28	2.88	2.81	18.62	9.28	2.01	14.57	38.43	9.92	3.87
32	SIB-7	2.87	12.56	3.57	3.52	3.46	15.71	5.54	2.83	10.84	31.17	11.37	2.74

The 35 *C. canephora* accessions were characterized for the various morphological and physiological traits under stress and non-stress moisture regime of the two experiments. Amongst 35 accessions under two pot experiments, six accessions having high root biomass with high WUE and high TDM with high WUE and three accessions with low root biomass, TDM and WUE were selected & presented in this table.

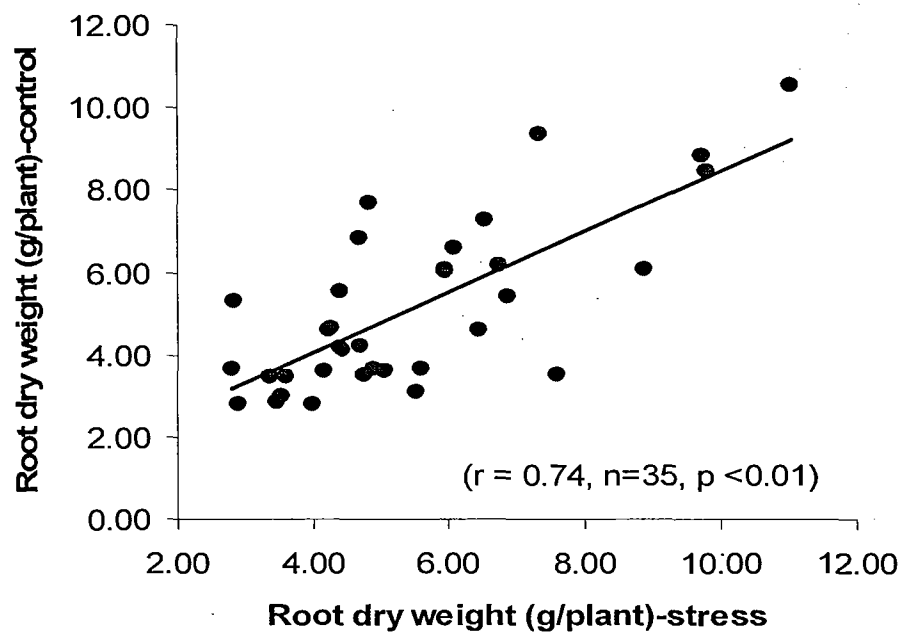


Figure 5. Root dry weight under stress (60% FC) in relation with non-stress (80%) of *Coffea canephora* accessions. In the first pot experiment 35 accessions were grown under two moisture regimes, i.e., 60% and 80% FC. The root dry weight was recorded at 160 days after transplanting into the pots. The root dry weight of all 35 accessions under 60% FC were plotted against 80% FC. A strong positive correlation was observed between root dry weight under stress and non-stress conditions.

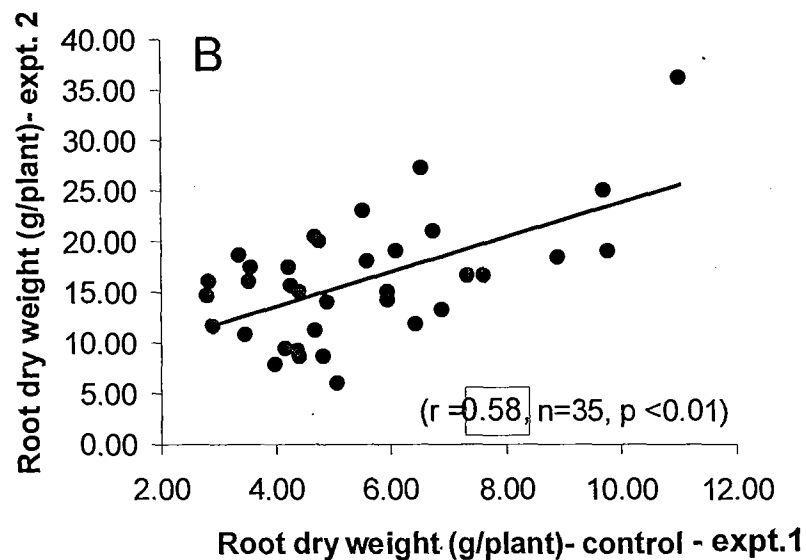
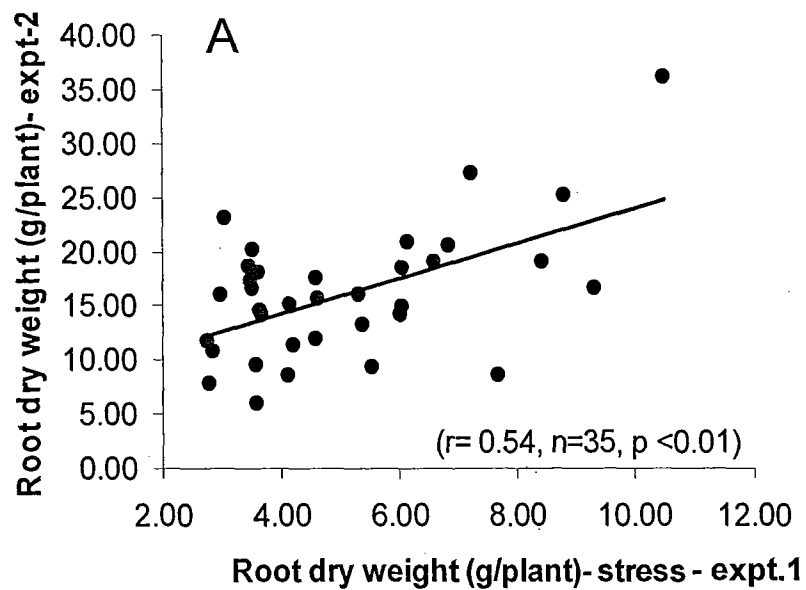


Figure 6. Relationship between root dry weight in two pot culture experiments. In the second pot experiment 35 accessions were grown under 80% FC. The genotypic variation in root dry weight under 80% FC of second pot experiment was plotted against the 60% FC (A) and 80% FC (B) of first pot experiment. At both the level of moisture regimes the correlation was positive and significant.

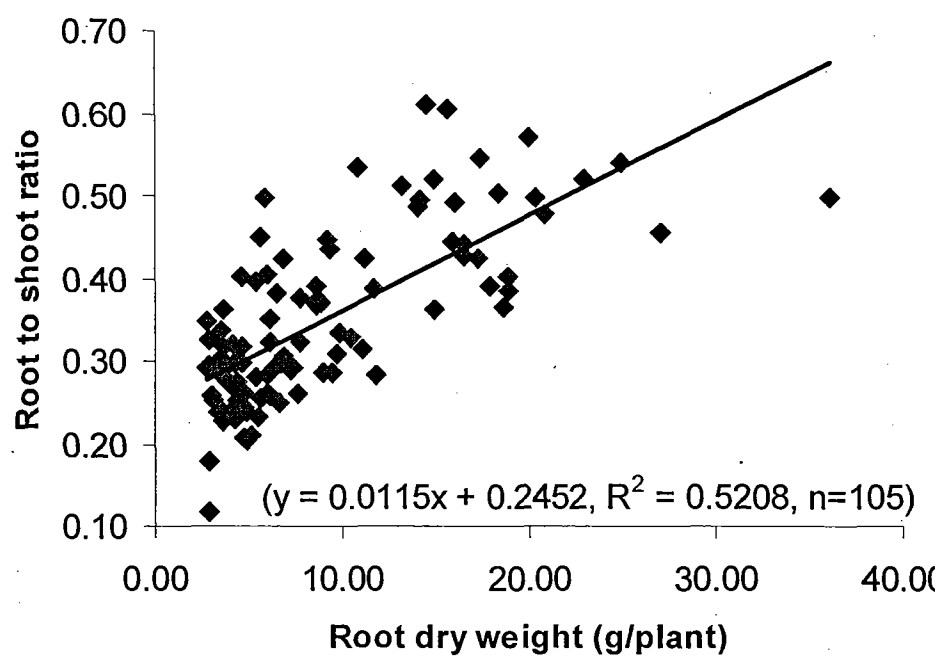


Figure 7. Relationship between root dry weight and root to shoot ratio under both experiments. From two pot experiments the root dry weight and root to shoot ratio was pooled and plotted. The correlation was positive and highly significant.

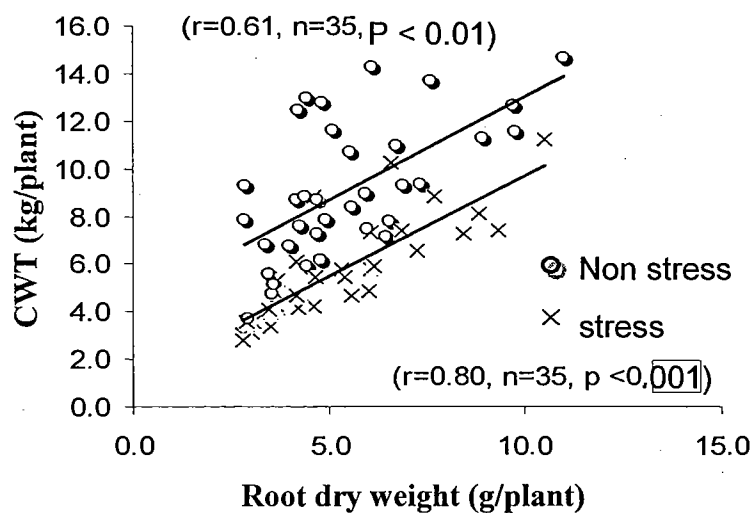


Figure 8. Relationship between root dry weight and cumulative water transpired under stress and non-stress conditions (experiment- 1).

In 35 accessions of *C. canephora*, the relationship between root dry weight at the end of the experiment and the total amount of water transpired was determined in two water regimes

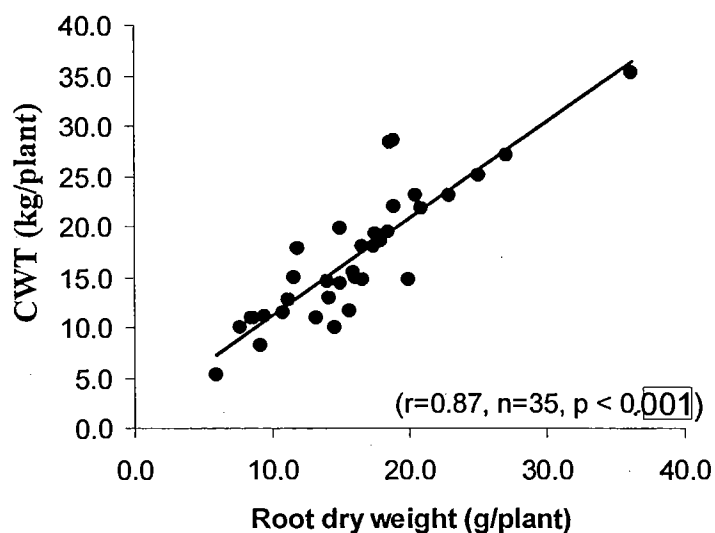


Figure 9. Relationship between root dry weight and cumulative water transpired under non-stress conditions (experiment- 2).

In the second pot experiment, 35 accessions were again raised under non-stress condition (80% FC) and root dry weight at harvest was plotted against CWT.

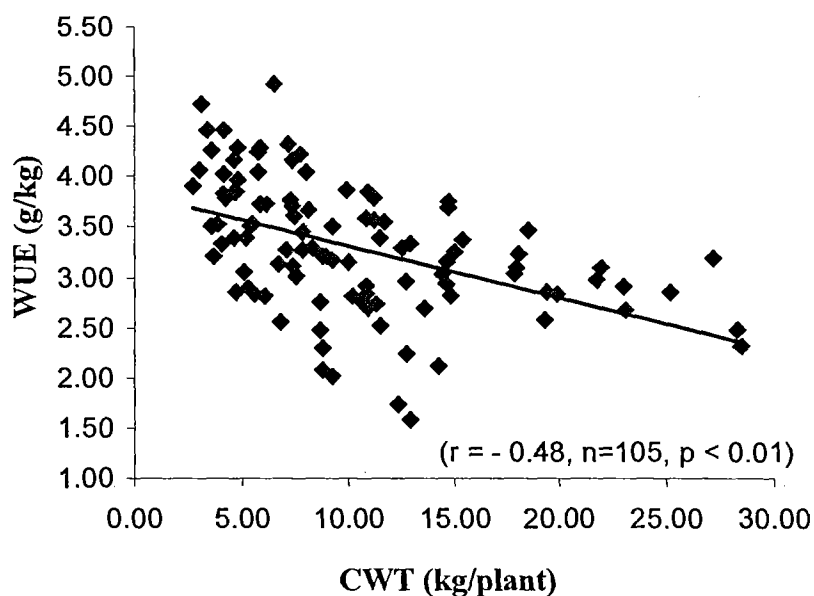


Figure 10. Relationship between CWT and WUE under first and second experiment.

The data of CWT from 80% & 60% of the first experiment and 80% FC of second experiment was pooled and plotted against the pooled data of WUE of first and second experiment. The relationship was significantly negative.

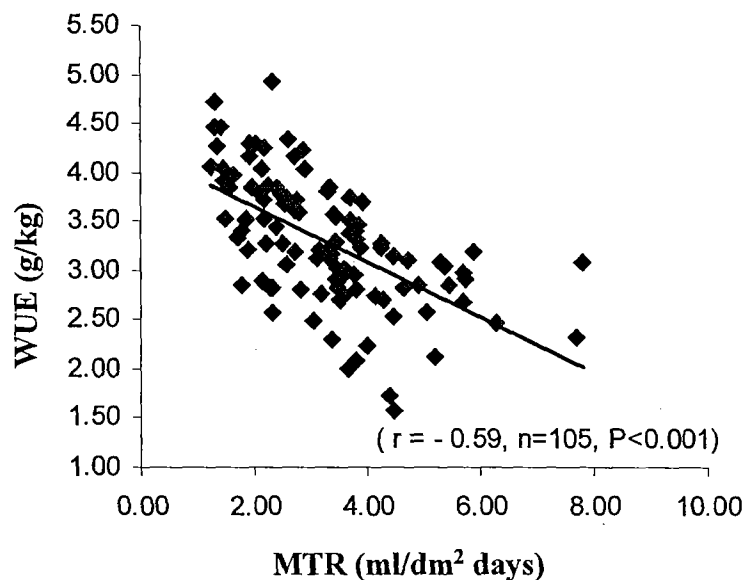


Figure 11. Correlation between MTR and WUE under first and second experiment. The MTR under first experiment and second experiment was pooled and plotted against the WUE. The relationship was significantly negative.

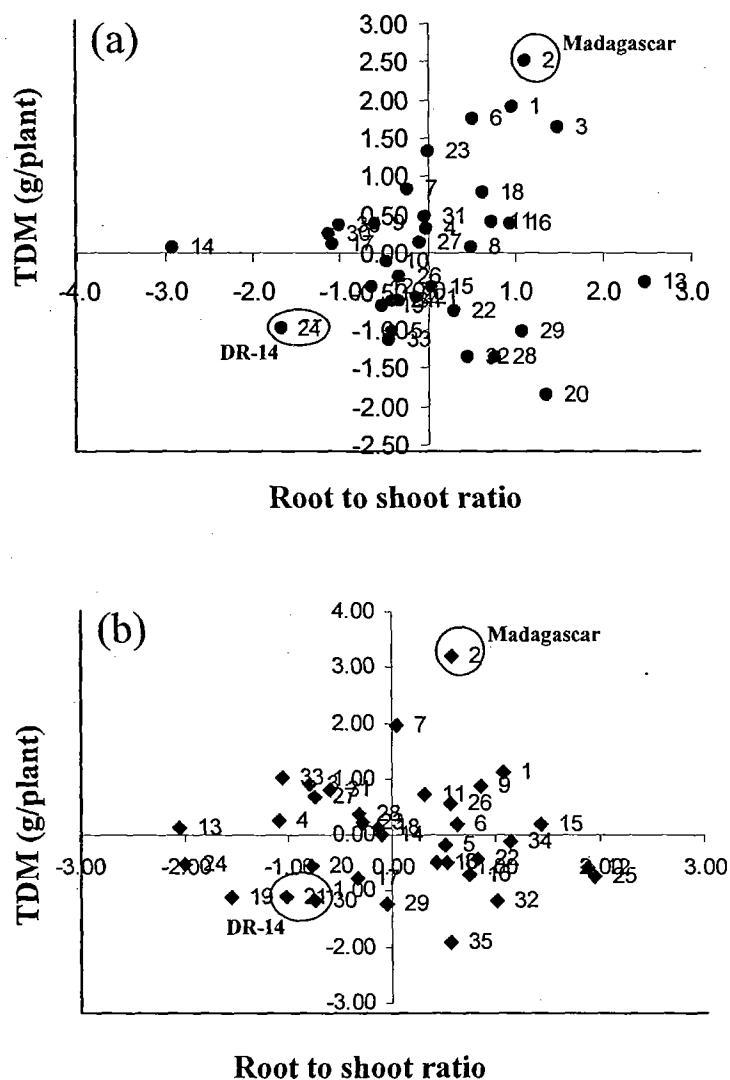


Figure 12. Standard normal distribution pattern of canephora accessions based on their root to shoot ratio and TDM. The normalized Z values were calculated by subtracting the individual values with total mean and divide them by standard deviation value. By this way Z normalized values were calculated for RSR and TDM. To identify the high and low root types, the normalized Z values of RSR values were plotted against TDM under non-stress of the first (a) and Second pot experiment (B). From both the pot experiments, Madagascar (2) and DR14 (24) were selected as high and low RSR types and further these were crossed and a mapping population was developed.

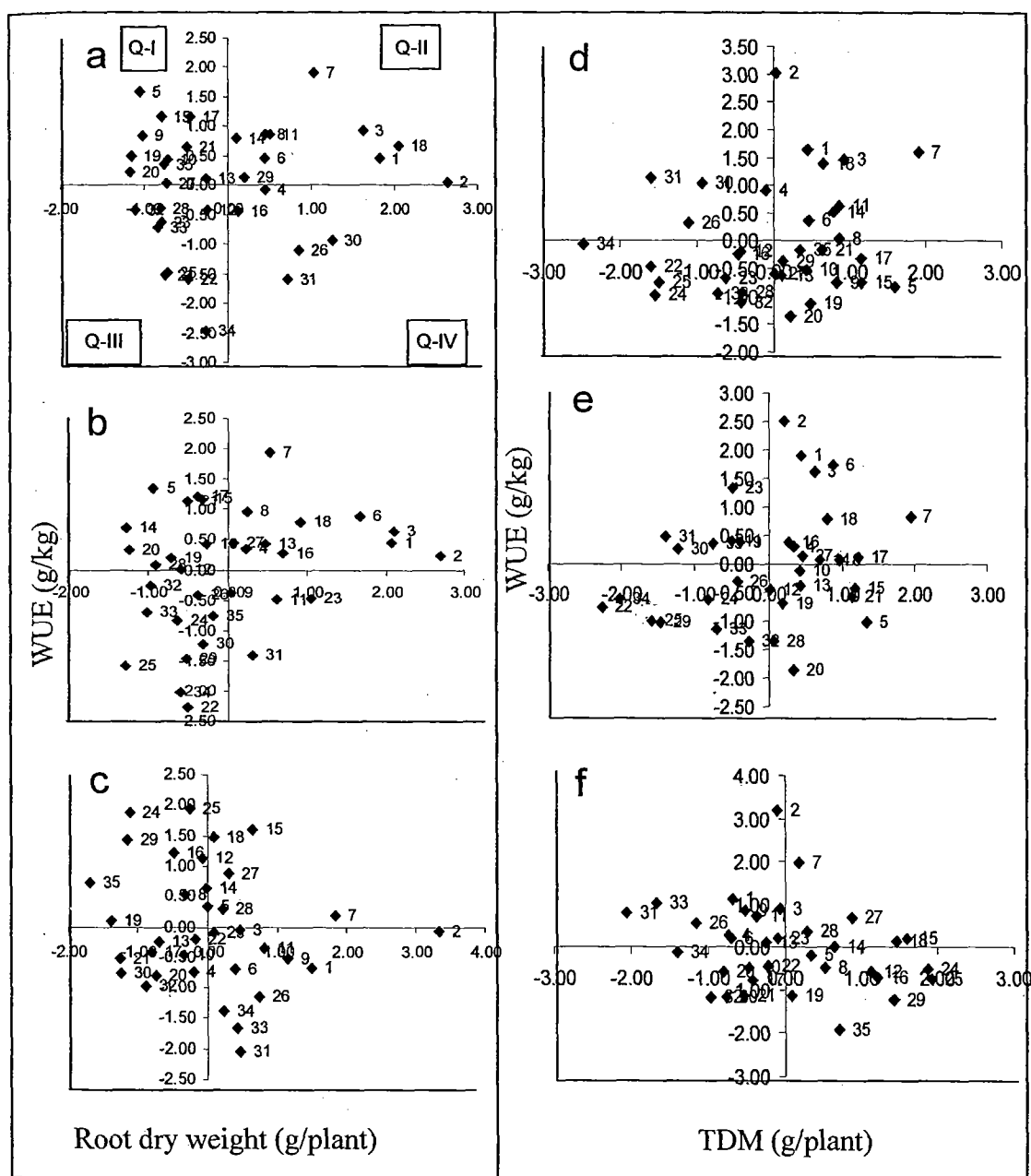


Figure 13. Standard normal distribution plot between root dry weight & WUE and TDM & WUE in *C. canephora* accessions grown under two pot experiments.

The graphs were plotted by using standard normal values of root dry weight, TDM & WUE. From the II quadrant of root dry weight v/s WUE under stress (a), non-stress (Expt-I) (b) and non-stress (Expt-2) (c), the high RDW & WUE types were selected. Similarly from the II quadrant of TDM v/s WUE under stress (d), non-stress (Expt-I) (e) and non-stress (Expt-2) (f), the high TDM & WUE types were selected. In contrast the low types were also selected from the III quadrant from two pot experiments.

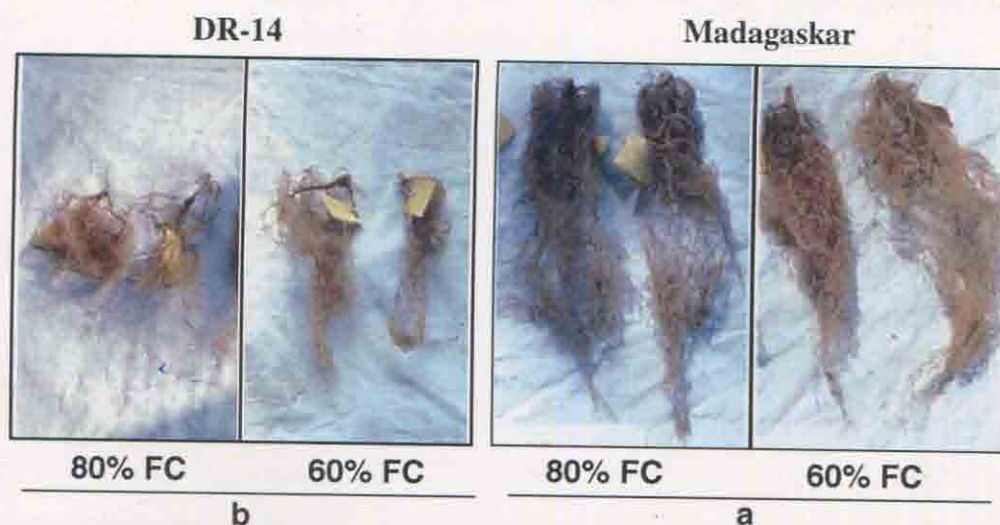


Plate 12: Typical root system of *Coffea canephora* accessions in experiment -1. The experiment was conducted at two levels of moisture regimes, i.e., 60% FC & 80% FC. Root dry weights were recorded at 160 days after transplanting into the pots.. Amongst the 35 accessions, Madagascar belongs to high root shoot ratio type (a) and DR-14 grouped into low root to shoot ratio type (b).



Plate 13: Typical root system of *Coffea canephora* accessions in experiment -2. The 35 accessions were once again quantified for the morpho-physiological traits through gravimetric measurements at 80% FC. In the first pot experiment the obtained accessions such as Madagascar as high and DR-14 as low root type were consistently existed in the second pot experiment. These two accessions were further crossed and developed mapping population.

4.4.3.2a Cluster analysis of the 35 *C. canephora* accessions using RAPD markers

The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. Using STASTICA software the scored binary data was employed to calculate genetic distance. Based on calculated genetic distance the linkage distance (dendrogram) was plotted.

The RAPD markers generated dendrogram of all 35 accessions showed three major clusters. The cluster analysis revealed maximum linkage distance of 95 units (Fig 14). The maximum linkage distance of the first cluster was 30 units, which included dwarf CxR, robusta high yield, robusta bold, Sib-7, 4, DR-18, 19, 20. These accessions belonged to low root to shoot ratio types. The second cluster linkage distance was 50 units, which included the moderate root to shoot ratio accessions such as, DR-3, 6, 7, 11 and 12. The linkage distance of third major cluster was 53 units, which contained four sub clusters. The first sub group of third major cluster included DR-10, 13, 14, 15, 16 & 17; which are relatively medium root to shoot ratio types. The second and third sub clusters contained ten accessions; they are relatively high root to shoot ratio types. Fourth sub group contained the high root to shoot ratio types like Java, Madagascar, DR-8, 9 and Saigon (Fig 14).

4.4.3.2b Dissimilarity matrix

The dissimilarity matrix constructed by squared Euclidean distances (SED) which estimated all the pair wise differences in the amplification product as explained by Sokal and Sneath (1973), revealed that the maximum dissimilarity of 31 units was observed between DR-8 and DR-3, followed by 30 units between DR-9 and Dr-4. The minimum dissimilarity of 2 units was observed between Dwarf CxR and Robusta high yield accessions followed by Robusta bold and robusta high yield with 5 units (Table 29). The clone DR-3 had more dissimilarity amongst the DR series from DR-4 to DR-20 (ranged from 19-28 units). Basically single clone did not obtain these DR accessions; however they were collected during drought conditions in different estates of Wynad district (Regional Coffee Research Station, Kerala state). Hence these DR accessions not

Table 28: Primers and their sequences used in the genetic diversity study

Sl.No	Primer	Sequence 5'- 3'	Total no. of bands	No. of Polymorphic bands
1	OPL18	ACCACCCACC	27	8
2	OPL2	ACTGAACGCC.	32	3
3	OPM13	GGTGGTCAAG	30	5
4	OPN16	AAGCGACCTG	21	15
5	OPN18	GGTGAGGTCA	32	3
6	OPN20	GGTGCTCCGT	30	5
7	OPO12	CAGTGCTGTG	24	11
8	OPO15	CCAAGCTGCC.	25	10
9	OPO5	GTGTCTCAGG.	22	13
10	OPO7	CAGCACTGAC	25	10
11	OPP6	GTGGGCTGAC	30	5
12	OPP7	ACACAGAGGG.	30	5
13	OPQ5	CCGCGTCTTG	17	18
14	OPQ15	GGGTAACGTG	28	9
15	OPR1	TGCGGGTCCT	23	12
16	OPT7	GGCAGGCTGT	27	8
17	OPV7	ACAGGTGCGT	31	4
		TOTAL	454	143

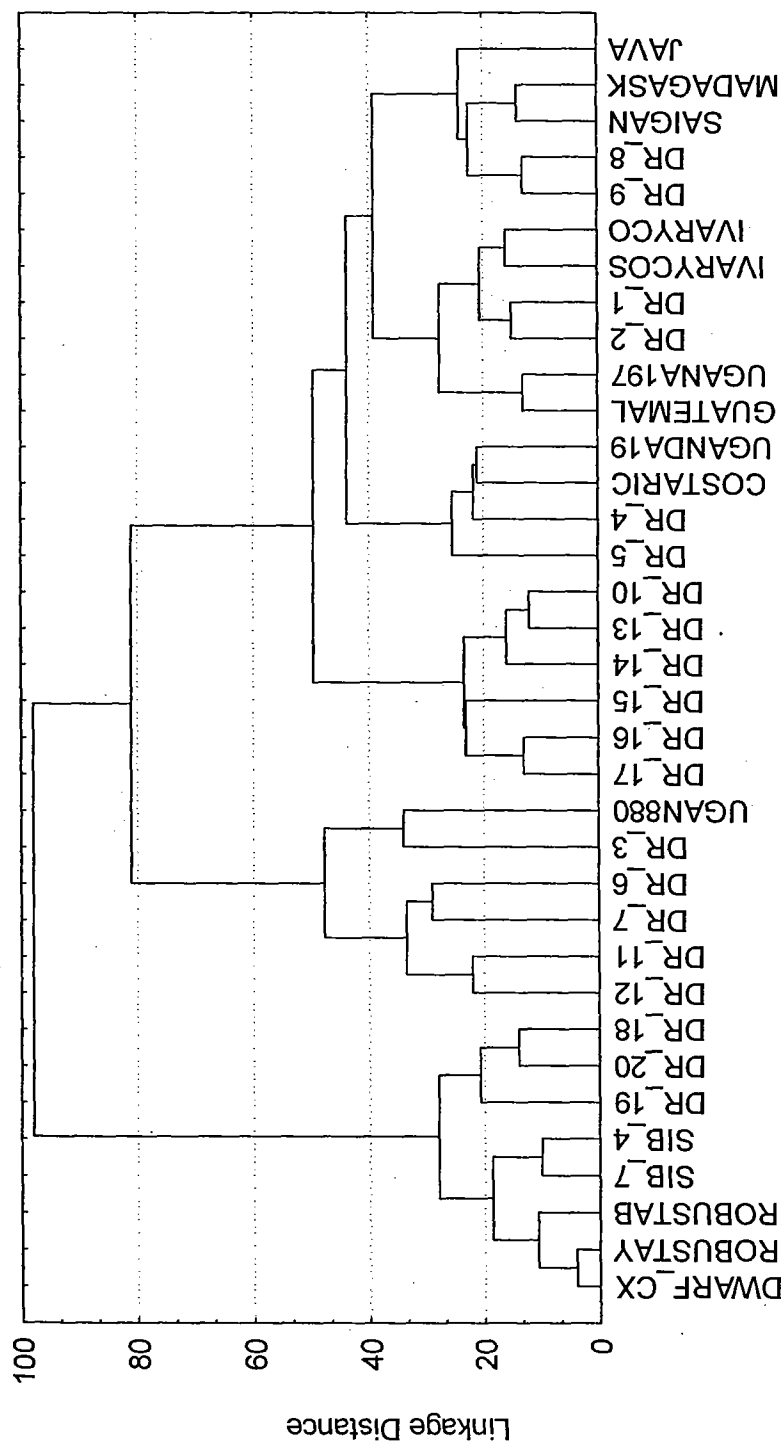


Figure 14. UPGMA dendrogram showing the genetic relationships among the 35 *Coffea canephora* accessions. These accessions were genetically characterized with 17 informative dominant RAPD markers and generated 454 bands. The bands were scored for presence 1 and absence 0. Finally the binary data was employed in statistical software and the genetic distance was drawn based on wards method.

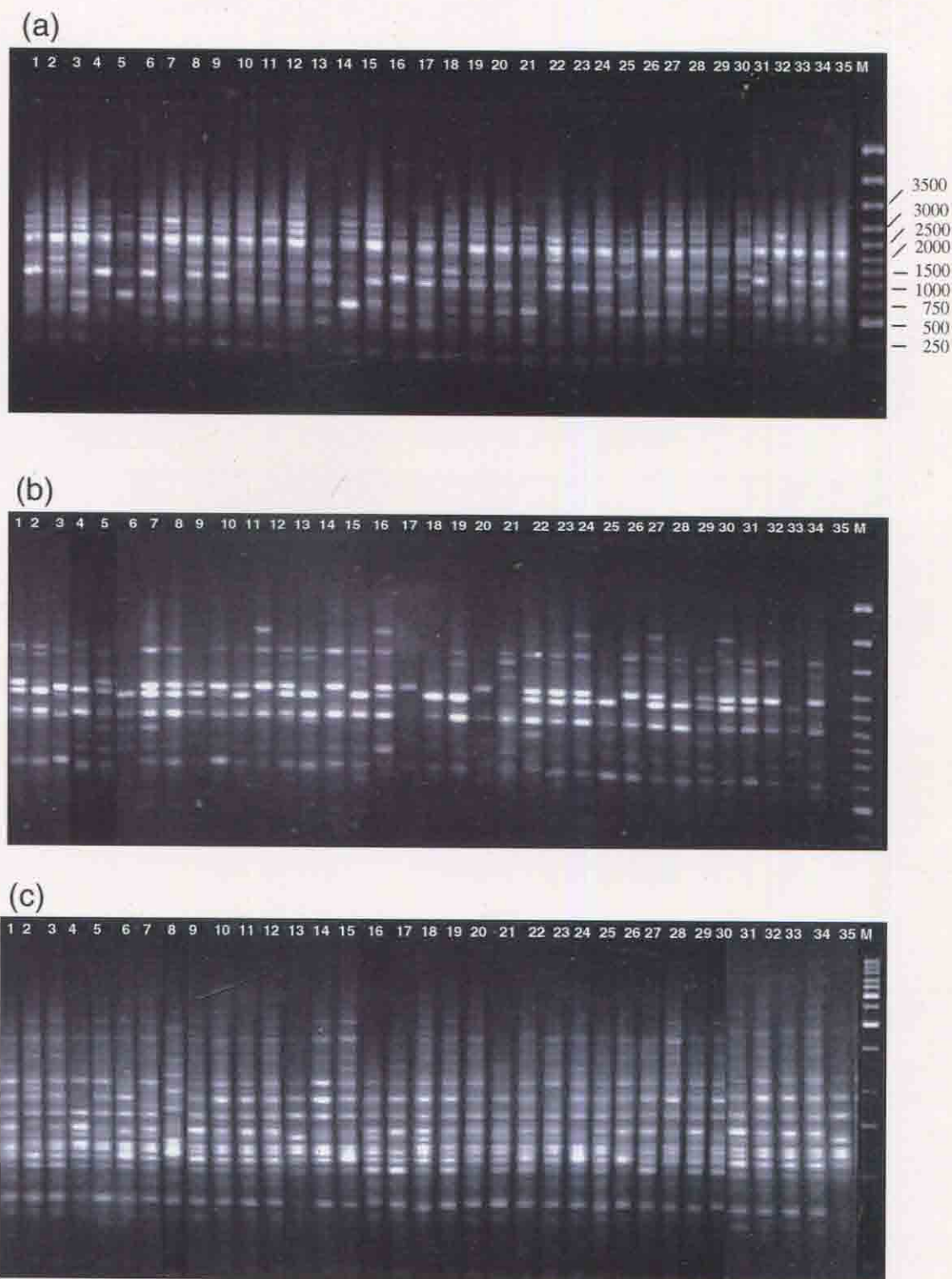


Plate 14a: RAPD gel profile of thirty five *Coffea canephora* accessions. To study the genetic diversity, the 35 *C. canephora* accessions were molecular characterized with RAPD markers. The gel profiles are the representatives of some RAPD primers, such as OPP6 (a), OPQ5 (b), OPT7 (c)

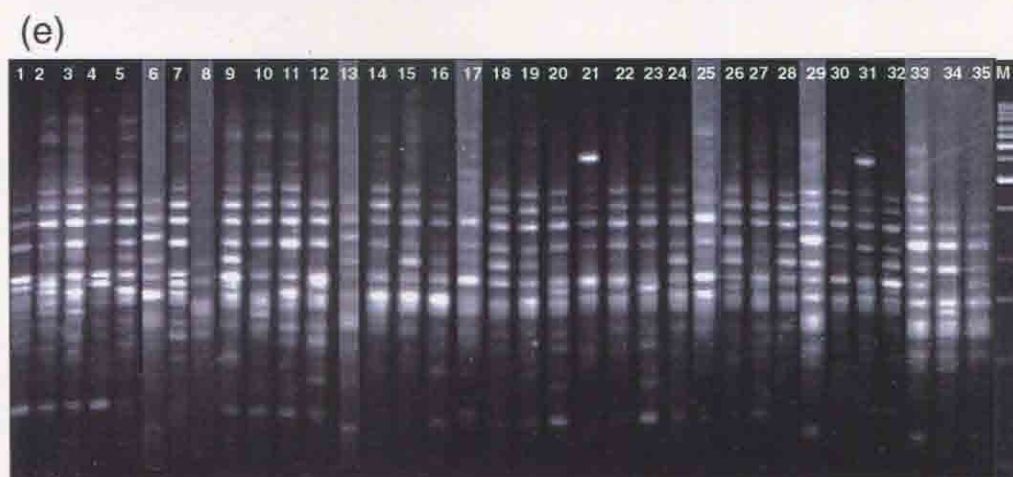


Plate 14b: RAPD gel profile of thirty five *Coffea canephora* accessions. To study the genetic diversity, the 35 *C. canephora* accessions were molecular characterized with RAPD markers. The gel profiles are the representatives of some RAPD primers, such as OPO15 (d), OPL18 (e), OPO12 (f)

descended by single parent. In the dendrogram it was noticed that all the DR accessions were not grouped together, rather they were grouped with other accessions.

Amongst 35 accessions, the selected high root to shoot ratio clone Madagascar had more dissimilarity units (28) with DR-3 and the low RSR type clone, DR-14 showed maximum dissimilarity (26 units) with DR-2. However the between Madagascar and DR-14 the dissimilarity was 12 units. Though they did not show much dissimilarity by genetic data, they are phenotypically very dissimilar with respect to root to shoot ratio. It was confirmed by two pot experiments.

4.4.3.3 Assessment of genetic diversity using morphological and physiological characteristics

The values of the physiological and morphological traits were normalized and the linkage distances were calculated as explained earlier. The generated dendrogram revealed maximum linkage distance of 5500 units (Fig 15) with two major clusters. The first cluster covered 1250 units and contained two sub clusters with 27 accessions, have low to medium root biomass types ranged from 2.0-7.0 g/plant with mean of 5.02 g/plant.

The second major cluster contained seven accessions with high root biomass ranging from 7.0-11.03 g/plant with mean of 7.67 g/plant. Especially Madagascar made separate group and had highest root biomass of 11.03 g/plant. In fact, the phenotypically selected accession DR-14 having low root type and Madagascar with high root type sits apart with a linkage distance of 700 units. However, in the genetic distance the distance between these two accessions was very low. In our study the insufficient markers may not explain their genetic distance sufficiently. Therefore some more markers like SSR and AFLP could still differentiate their genetic distance.

In comparison with phenotypic distance, the high root types were not made separate group in the genetic distance, instead they made a separate sub cluster along with medium root biomass types. This indicates though they are phenotypically distinct, but genetically not significantly different.

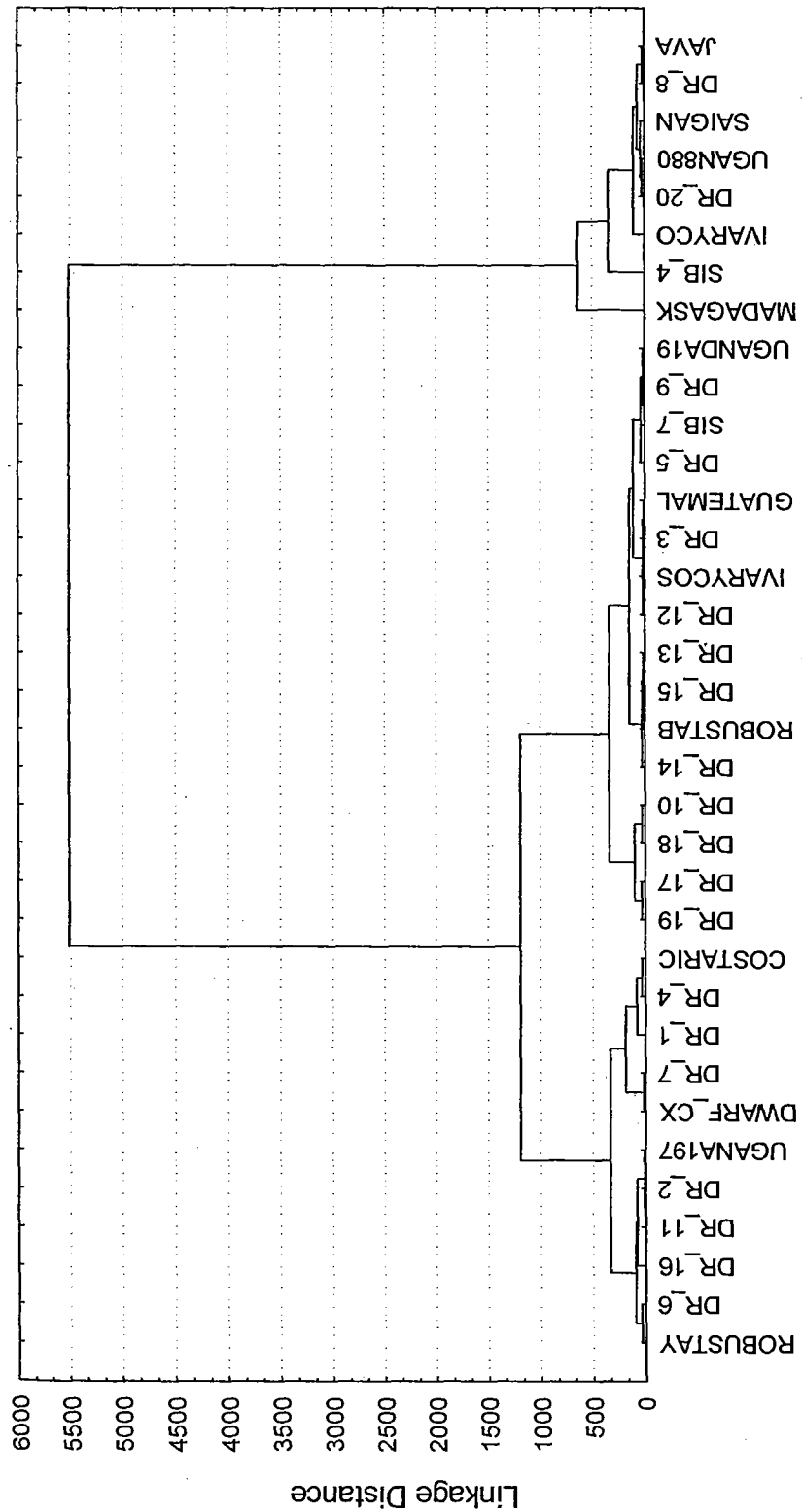


Figure 15. UPGMA dendrogram showing the morpho-physiological relationships among the 35 *Coffea canephora* accessions. The 35 accessions were phenotypically characterized for more than ten traits, the recorded numerical trait values were analyzed in statistical software and linkage distance was plotted.

4.4.3.3a Principle component analysis (PCA)

Based on RAPD markers the three dimensional PCA formed three groups. The first group consists of high root types like Java, Madagascar, Saigon, Ivorycost, Costarica, and Guatemala. In these accessions root biomass ranged from 6.0-11.0 g/plant, with a mean of 7.78 g/plant. The second group contained low and medium root types, the value ranged from 4.0-5.0 g/plant. However the third group had accessions like DR-3, 6, 7, 11 and Uganda 880, which were placed distinctly in the analyzed PCA (Fig. 16a).

The three dimensional PCA based morphological data revealed existence of large variation among the accessions. It depicted clusters observed in the phenotypic dendrogram. However the Saigon, DR-5, 7 and 9 made different group, belonged to medium root biomass type (Fig 16b).

4.4.3.3b Comparison of dendrogram and PCA generated by RAPD and physiological & morphological traits

A maximum linkage distance of 5500 units was observed in morphological cluster (Fig 15) where as a maximum of 95 units were noticed in dendrogram based on RAPD data (Fig 14). It revealed a genetically low variation among these accessions even though a large variation was noticed in the morphological and physiological traits. Similar results were also noticed in the dissimilarity matrix constructed by using RAPD (Table 29).

A well dispersion of the points in the three-dimensional PCA based on physiological traits revealed large variability for the traits studied in this investigation (Fig 16b). Where as very tight and close distribution of the accessions noticed in three-dimensional PCA based on RAPD data (Fig 16b) revealed a narrow genetic diversity. The correlation between dissimilarity matrices generated by these methods ($r = 0.3$) indicated they are positively associated but the relationship was non-significant. So observed physiological and morphological differences in these requires large number of genetic markers (RAPD) for significant correlation. It suggested requirement for much more number of markers as well as morphological traits to be used to distinguish various groups.

Table 29: Dissimilarity matrix constructed by Squared Euclidean distance using RAPD marker data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	
1 JAVA	0																																			
2 MADAGASK	12	0																																		
3 SAIGAN	12	8	0																																	
4 UGAN880	15	21	19	0																																
5 UGANDA19	13	12	11	17	0																															
6 COSTARIC	16	14	15	19	12	0																														
7 IVARYCO	15	15	15	21	17	12	0																													
8 UGANA197	12	13	12	21	14	13	10	0																												
9 GUATEMAL	17	12	14	23	14	11	15	8	0																											
10 IVARYCOS	14	16	15	23	18	15	9	10	11	0																										
11 DR 1	12	13	13	20	15	12	10	9	14	10	0																									
12 DR 2	13	14	12	22	17	15	11	10	15	12	9	0																								
13 DR 3	23	28	28	20	23	21	27	28	30	29	24	28	0																							
14 DR 4	17	17	15	19	12	13	16	15	17	16	15	16	19	0																						
15 DR 5	18	14	15	21	14	15	15	12	14	17	12	14	27	14	0																					
16 DR 6	18	22	22	21	18	20	21	21	22	21	20	20	18	16	18	0																				
17 DR 7	15	14	15	24	17	18	17	17	18	17	15	16	24	18	17	17	0																			
18 DR 8	14	12	12	24	15	17	15	11	13	11	12	14	31	18	17	24	16	0																		
19 DR 9	11	8	9	20	14	17	15	9	12	11	9	12	30	17	14	21	17	8	0																	
20 DR 10	15	13	12	20	17	14	14	14	17	15	13	8	25	15	17	20	18	15	12	0																
21 DR 11	16	20	20	20	18	17	22	18	23	22	15	20	19	20	21	15	19	20	15	18	0															
22 DR 12	18	17	17	22	20	17	16	14	17	14	14	16	27	19	17	20	17	14	13	13	0															
23 DR 13	16	12	12	21	16	13	10	14	14	12	11	10	28	18	15	22	17	12	9	7	19	13	0													
24 DR 14	15	12	12	22	15	14	12	11	14	11	11	12	26	15	11	20	14	12	9	9	18	14	8	0												
25 DR 15	17	15	18	25	15	14	18	15	15	15	12	14	24	20	17	20	20	15	15	14	15	11	11	0												
26 DR 16	17	14	14	22	15	14	15	15	15	16	13	12	27	18	17	22	18	12	11	11	19	17	9	12	12	0										
27 DR 17	15	14	14	22	15	15	14	14	15	15	11	12	25	18	14	19	16	14	11	11	18	15	9	10	12	8	0									
28 DR 18	20	17	16	25	18	17	19	17	20	18	15	18	27	17	21	19	18	14	14	14	17	15	15	14	15	9	9	0								
29 DR 19	20	18	18	25	18	15	18	17	21	20	15	18	24	18	18	21	15	15	19	18	17	14	17	15	14	17	16	12	0							
30 DR 20	18	15	14	24	15	14	15	14	17	15	14	14	25	17	17	19	15	12	14	14	17	11	12	15	12	14	9	8	10	0						
31 SIB 4	22	18	20	28	21	17	18	18	19	18	17	19	28	23	21	24	17	15	18	17	20	14	16	16	13	14	11	11	10	9	0					
32 SIB 7	20	15	17	24	18	15	17	17	15	15	17	25	20	18	20	15	13	16	15	20	14	12	14	9	10	9	11	10	8	6	0					
33 ROBUSTAB	18	15	17	24	18	12	15	15	17	15	12	15	27	20	17	21	15	13	14	15	18	15	14	12	12	11	9	11	12	9	8	6	0			
34 ROBUSTAY	17	14	14	24	16	14	15	15	16	14	14	15	26	18	17	18	14	10	12	12	17	12	9	12	10	9	9	10	12	8	9	5	5	0		
35 DWARF CX	17	14	15	24	17	14	17	17	16	15	15	16	26	20	17	18	13	11	13	13	17	12	12	12	11	11	11	12	12	10	7	5	2	0		

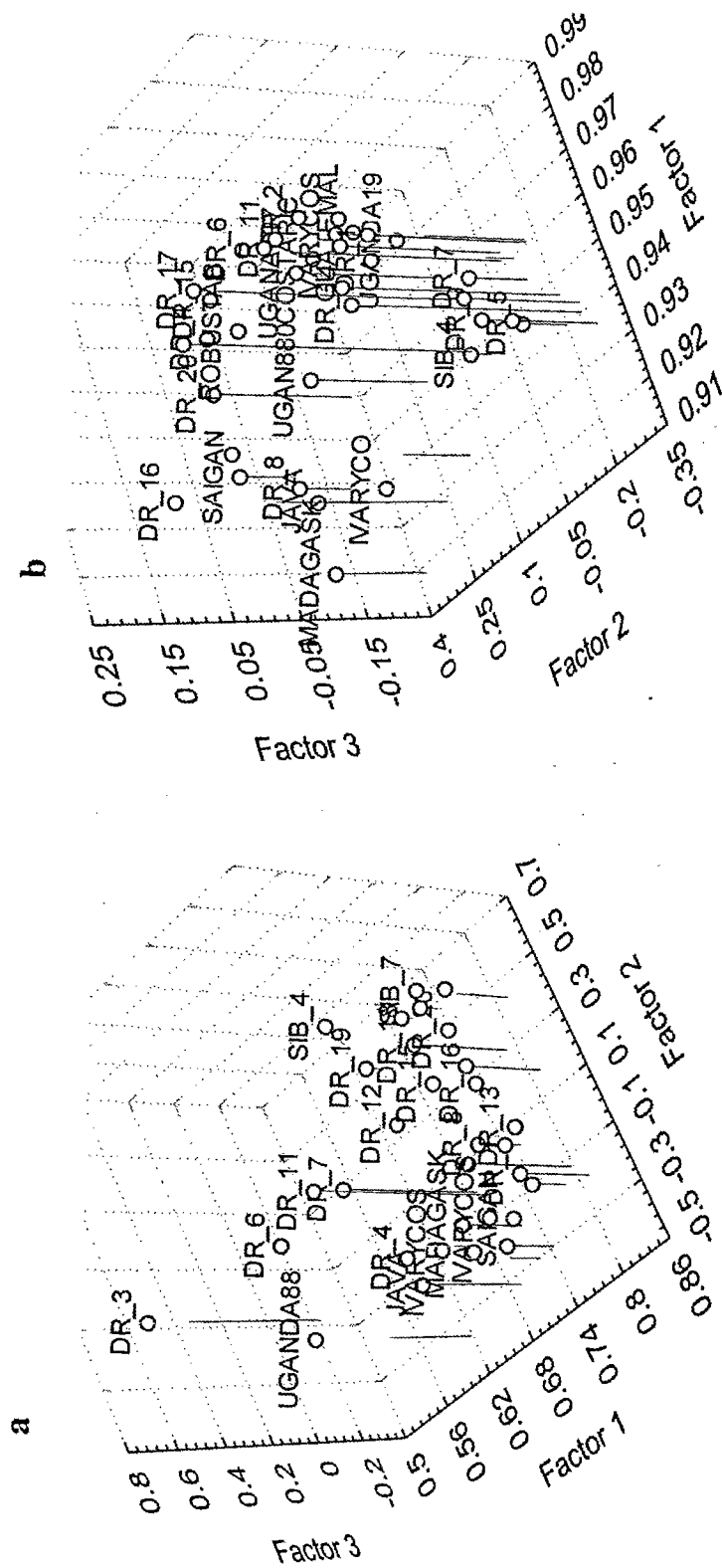


Figure 16: Principle coordinate analysis (PCA) of genetic distance among 35 *C. canephora* accessions. (a) PCA was developed by using the RAPD markers, three major coordinates explain high, medium and low root to shoot ratio types. (b) PCA was developed by using phenotypic traits.

Table 30: Range and mean for the morphological and physiological traits in the mapping population of (DR-14 x Madagascar) *C. canephora* accessions

	Parameters	Range	Mean	SD
1	Plant Height (cm)	31.00- 83.00	60.46	9.61
2	No. of nodes	8.00- 41.00	20.92	6.33
3	No. of primaries	1.0- 10.00	5.10	2.28
4	No. of Leaves/plant	16.00- 77.00	40.48	12.08
5	Leaf area initial (cm ² /plant)	94.59-1124.42	668.55	189.53
6	Leaf area final (cm ² plant)	1371.24-9273.08	4241.66	1327.31
7	Stem Girth (mm)	5.66- 12.68	10.37	9.25
8	Root Length (cm)	31.00-75.00	51.93	9.66
9	Leaf dry weight (g/ plant)	4.89-41.91	19.38	7.11
10	Stem dry weight (g/ plant)	3.19-33.21	14.52	5.51
11	Shoot dry weight (g/ plant)	10.55-75.12	33.90	12.25
12	Root dry weight (g/ plant)	2.87-22.42	9.24	3.41
13	Root to shoot ratio	0.13-0.56	0.28	0.06
14	Total biomass (g/ plant)	14.10-89.48	43.14	15.28
15	Differential total biomass (g/ plant)	2.20-77.58	31.20	15.34
16	cumulative water transpired (kg/plant)	3.21-20.69	10.80	3.69
17	Water use efficiency (g/kg)	0.69-4.32	2.76	0.71
18	Leaf area duration (dm ² days)	2055.75-13369.27	6604.23	1933.59
19	Net assimilation rate (mg/ dm ² days)	0.72-7.43	4.47	1.26
20	Mean transpiration rate (ml/ dm ² days)	0.88-2.61	1.64	0.31

4.4.4.1 Development of mapping population of *Coffea canephora* (DR-14 x Madagascar)

Based on phenotypic and genetic analysis the contrasting accessions with six high and three low root biomass types were crossed with all possible combinations. However except DR-14 having low root biomass and Madagascar with high root biomass cross, all other cross combinations showed very poor seed set. The DR-14 and Madagascar combination cross, produced more than 150 fruits and were carefully collected after full maturity and sown in the nursery bed. Of the collected 150 seeds, only 127 seeds could germinate in the nursery bed. Further these seedlings were transferred to poly bags and after six months they were transplanted in to containers holding 35 kg of soil. One month after transplanting, these planted pots were maintained at a water regime of 70% FC. To study the parental segregating characters in the 127 F1 mapping population, the individuals were characterized through gravimetric approach for several physiological and morphological traits.

4.4.4.2 Phenotypic analysis of mapping population of *Coffea canephora* (DR-14 x Madagascar)

4.4.4.2a Gravimetric analysis to assess root traits, WUE and related physiological traits in mapping population

The variability in roots and other physiological traits were assessed at a single moisture regime of 70 % FC by gravimetric approach. This approach apart from giving data on root traits also provided an option to quantify physiological traits like total transpiration, NAR, MTR and WUE.

Significant variability with respect to morphological traits was noticed in the F1 mapping population. The maximum plant height varied from 31.0 (MP 131) -83.0 cm (MP 8). The average length was 60.46cm. Similar variations were noticed in no. of nodes (8.0 - 41.0), no. of primary branches (2 -10) and no. of leaves (16 - 77). Similarly in the case of stem girth varied from 5.66-12.68 mm, however the leaf area showed a well-marked difference from 1371– 9273 cm, with an average of 4251 cm (Table 30). The quantitative nature of these traits was clearly elucidated in Fig 17. This indicates the effective crossing was

taken place between two parents, this indicates no bias of single parental characters in the developed mapping population.

4.4.4.2b Root length & Root dry weight (RDW)

The root length and dry weight was recorded at 269 days after transplanting into the pots. In the Mapping population, the maximum root length varied from 31.0-75.0 cm, with an average of 51.93 cm and root dry weight ranged from 2.87 to 22.42 g/plant with mean of 9.2 g. This kind of variation indicates that these traits are quantitative in nature. The frequency distribution of root dry weight was normally distributed (Fig 17) and 58% of the individuals have root dry weight of 5-10 g/plant. The root dry weight of 127 F1 mapping population is presented in Appendix – II.

4.4.4.2c Shoot dry weight (SDW)

The shoot dry weight includes stem and leaf dry weights recorded individually (Appendix -II). The SDW ranged from 10.55 (MP 73) -75.12 (MP16) g/plant with a mean of 33.9 g (Table 30). Interestingly the mapping individual 73 has low leaf area showed low shoot dry weight and high leaf area individual 16 has high shoot dry weight (Appendix-II). This indicates the shoot dry weight is strongly dependent on leaf area and hence the relationship between leaf area and shoot dry weight is positive and highly significant (Fig 19). The relationship between root dry weight and shoot dry weight was positive ($r=0.86$) (Fig 18). A significant variation of shoot dry weight in 127 F1 progenies showed quantitative nature of the trait (Fig 17).

4.4.4.2d Total dry matter (TDM)

The total dry matter accumulation varied from 14.10 – 89.48 g/plant with a mean of 41.54 g. The frequency distribution of 127 individuals with respect to total dry matter is given in Fig 12. It was observed that 53.0% of the individuals accumulated 30-50g/plant of total biomass. A strong positive significant relationship was noticed between total dry matter and Leaf area ($r= 0.91$) and leaf area duration ($r=0.92$) (Table 31).

It also noticed that the leaf area is positively correlated with NAR ($r=0.58$). This indicates the TDM is strongly dependent on the leaf area and leaf area duration. However a

Table 31: Correlation matrix of the morphological and physiological characters in the mapping population (DR-14 x Madagascar) of *C. canephora* accessions

	SDW	RDW	TDM	CWT	WUE	LA	LAD	NAR	MTR
SDW	1.00								
RDW	0.82	1.00							
TDM	0.99	0.89	1.00						
CWT	0.90	0.88	0.93	1.00					
WUE	0.74	0.54	0.72	0.43	1.00				
LA	0.91	0.75	0.91	0.85	0.63	1.00			
LAD	0.92	0.77	0.92	0.85	0.65	0.99	1.00		
NAR	0.82	0.79	0.84	0.77	0.68	0.56	0.58	1.00	
MTR	0.19	0.35	0.23	0.48	-0.27	-0.02	-0.02	0.50	1.00

Note: - The correlation values of > 0.325 are significant at 5% and values of > 0.418 are significant at 1%

Legend

SDW - Shoot dry weight, RDW – Root dry weight, TDM – Total dry matter,
 CWT – Cumulative water transpiration, WUE – water use efficiency,
 LA – Leaf area, LAD – Leaf area duration, NAR – Net assimilation rate,
 MTR – Mean transpiration rate

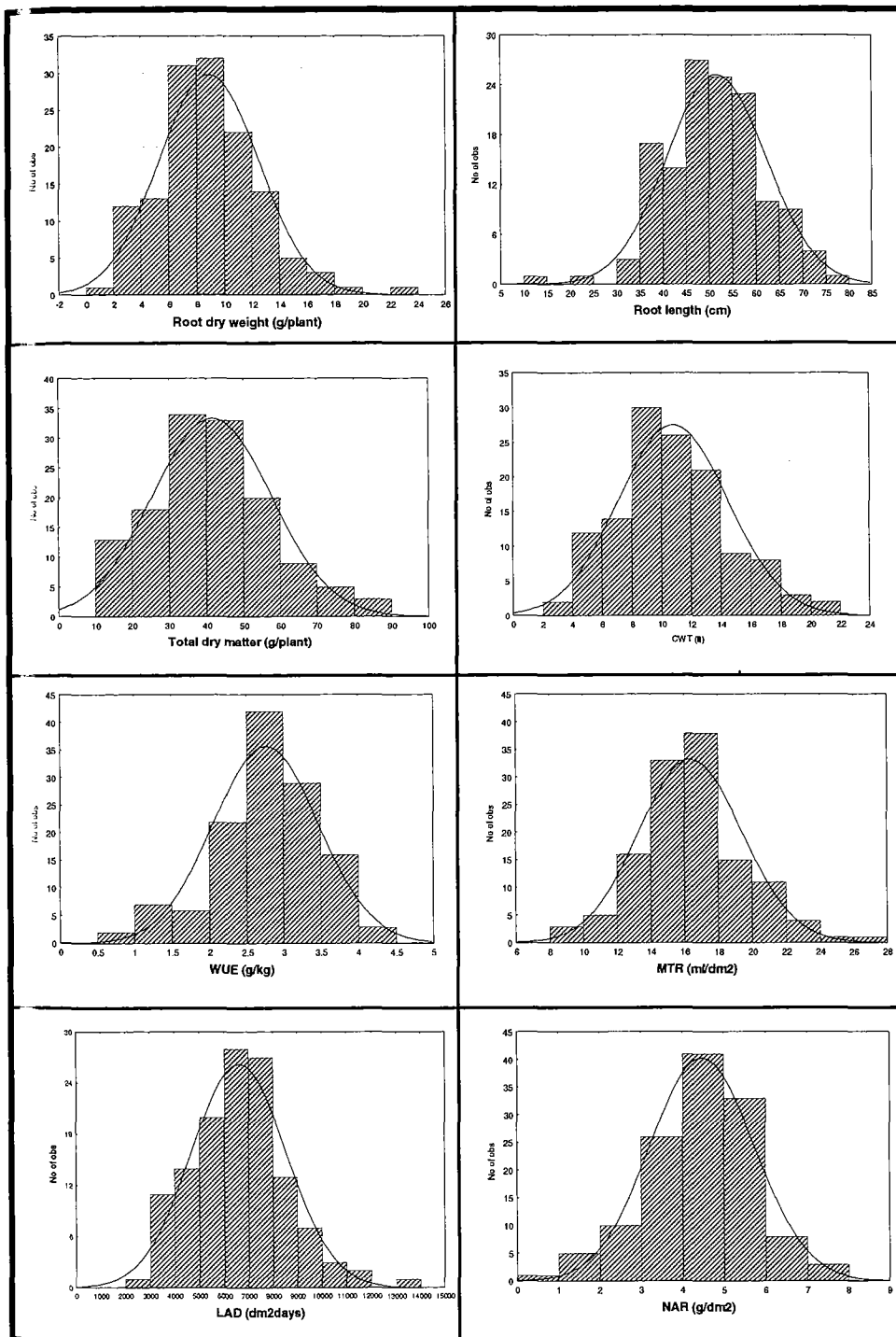


Figure 17: Frequency distribution of morphological and physiological characters in mapping population (DR-14 x Madagascar) of *C. canephora* accessions.

The mapping population developed from DR-14 x Madagascar were characterized for several phenotypic traits. The frequency of the some important traits were plotted and they showed the quantitative nature of the traits.

significant positive relationship between root dry weight and TDM ($r=0.89$) (Fig 18) signifies the increased TDM is also contributed by the increased root dry weight.

4.4.4.2e Physiological analysis of mapping population of *Coffea canephora* (DR-14 x Madagascar)

The cumulative water transpired varied from 3.21-20.69 kg/plant. The root dry weight is responsible for the more transpiration. Under stress condition root signifies its importance to harness the water from deeper profiles of soil. A positive relationship between RDW and cumulative water transpired was noticed ($r= 0.89$) (Fig 18). This strong interrelationship indicates that the total transpiration is influenced by root dry weight.

In the mapping population positive relationship between TDM and CWT ($r=0.93$) was noticed (Fig 20). This corroborates the conductance is directly associated with total dry matter.

However the water use efficiency as well showed a good amount of variation in the mapping population. It varied from 0.6-4.32 g/kg of water transpired. From our data we could identify a few individuals (MP 18, 20, 74 & 86) with high WUE over the parents (Appendix - II). The relationship between WUE and CWT ($r=0.73$), TDM and LAD ($r=0.92$) and net assimilation rate (0.72-7.43 mg/dm² days) and leaf area duration ($r=0.69$) were positive. However the mean transpiration rate showed negative relation to WUE ($r=- 0.24$) (Table 31) and relationship between MTR and leaf area was also negative (Fig 21).

4.4.4.3 Analysis of the locus specific SCAR markers in the mapping population (DR-14 x Madagascar)

In this study we have developed SCAR markers such as SCARL1 and SCARD1 which respectively linked to low root length and low root dry weight locus. These markers were initially validated in the parents (S.3334 and L1 valley) and mapping population, which was used to develop these markers. The amplification was positive in both low root length and low root dry weight parent (Plate 15). To test these two markers in the mapping population, 10 low & high root length and root dry weight individuals are selected from 134 individuals (Table 32). These 10 low and high root length individuals

were amplified with SCARL1 primer; the result indicated that only 7 low root length and 6 high root length individuals showed amplification (Plate 16). Similarly the SCARD1 amplified with 7 low root dry weight and 3 high root dry weight individuals (Plate 17). This indicates the associated marker may not be tightly linked to the trait.

Again these two markers are validated in the other two contrasting parental clones; DR-14 (low root length and root dry weight) and Madagascar (high root length and root dry weight), which have been selected based on the gravimetric approach. Here the amplification was positive in both low root length and low root dry weight parents (Plate 18).

Before validating SCAR markers in the mapping population, the specific markers were validated in another two contrasting parents; DR-14 (low root length and root dry weight) and Madagascar (high root length and root dry weight), which have been selected based on the gravimetric approach. The low root length linked SCARL1 was amplified in the low and high root length parents (DR-14 x Madagascar). The amplification showed a positive band in the low root length parent (Plate 18a). Whereas, in the case of root dry weight associated marker SCARD1 also showed positive band in the low root dry weight parent (Plate 18b).

These parents were crossed and obtained 127 F1 individuals, amongst them ten extreme low and high root length individuals were selected (Table 33). The selected individuals were amplified with SCARL1 primer. A single, distinct and identifiable band was amplified in both the low and high root length individual at approximately 850bp (Plate 19). This indicates that regardless of the significance level used, probability that a marker is false, given that a marker has been significantly associated with trait.

Similarly the marker linked to low root dry weight SCARD1 was amplified with ten extreme low and high root dry weight individuals. However the marker showed amplification in seven individuals of low root dry weight and rest three individuals were not amplified. Whereas high root dry weight individuals also showed amplification, but not comparable to the low root dry weight individuals (Plate 20). This indicates the marker is not tightly associated with trait.

Table 32: Classification of low & high root length and root dry weight F1 mapping individuals (L1 valley x S.3334)

	Sl. no	Plant No.	Root length (cm)		Plant No.	Root dry weight (g/plant)
Low root length types	1	8	41.50	Low root dry weight types	73	2.57
	2	16	46.0		33	3.76
	3	130	46.0		59	3.97
	4	86	46.50		130	3.25
	5	11	47.0		69	4.20
	6	33	47.0		5	4.69
	7	138	47.0		8	4.76
	8	34	47.50		11	4.87
	9	129	47.50		56	4.85
	10	1	48.0		16	5.19
High root length types	1	121	71.0	High root dry weight types	136	17.17
	2	4	71.50		3	24.42
	3	82	82.0		4	25.92
	4	77	72.50		6	27.09
	5	37	73.0		23	22.71
	6	100	74.0		53	25.56
	7	79	75.50		114	24.1
	8	40	76.0		105	30.09
	9	32	56.0		106	30.5
	10	72	62.0		121	30.82

Table 33: Classification of low & high root length and root dry weight F1 mapping individuals (DR-14 x Madagascar)

	Sl. no	Plant No.	Root length (cm)		Plant No.	Root dry weight (g/plant)
Low root length types	1	39	15	Low root dry weight types	103	2.57
	2	105	35		135	2.67
	3	17	35		136	2.84
	4	24	36		132	2.87
	5	27	37		134	2.98
	6	103	38		139	3.16
	7	83	38		133	3.62
	8	42	38		65	3.87
	9	33	38		52	4.11
	10	30	38		117	4.54
High root length types	1	1	64	High root dry weight types	45	13.51
	2	15	65		3	13.60
	3	18	66		11	13.71
	4	20	67		44	14.05
	5	14	67		112	14.27
	6	37	68		114	15.04
	7	112	69		18	16.18
	8	67	69		15	16.6
	9	135	70		10	17.31
	10	128	70		49	19.09
	11	131	70		51	22.42

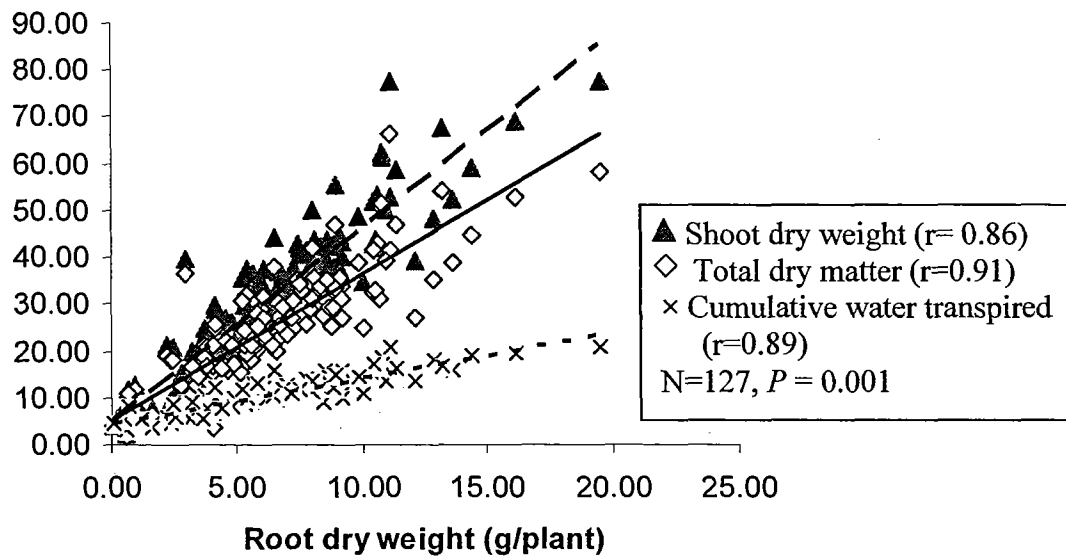


Fig 18. Relationship between root dry weight with shoot dry weight, total dry matter and cumulative water transpired.

In one hundred twenty seven F1 mapping individuals of *C. canephora* under 70% FC condition. The relationship between these traits are highly significant and positive.

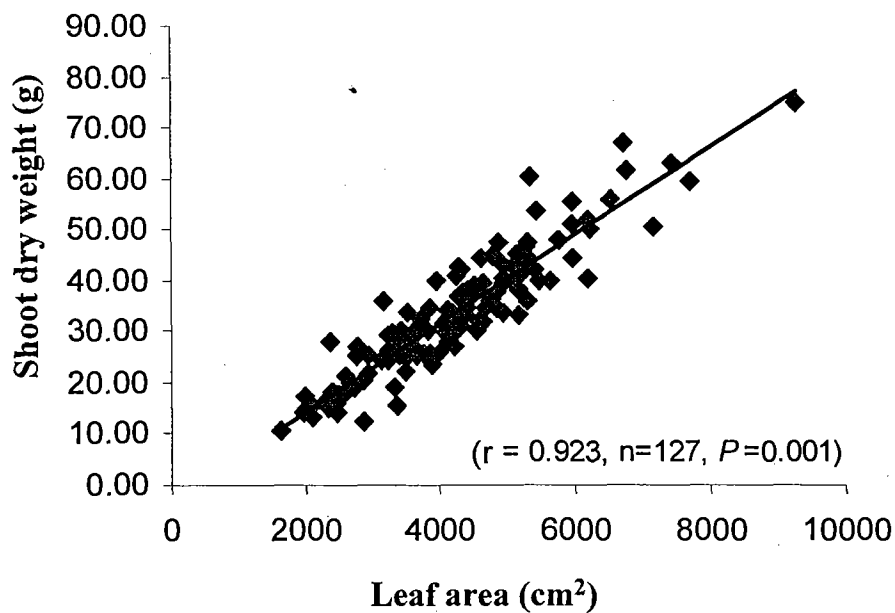


Fig 19. Relationship between leaf area and shoot dry weight in *C. canephora* mapping population. The 127 F1 individuals showed positive and highly significant relation between leaf area and shoot dry weight.

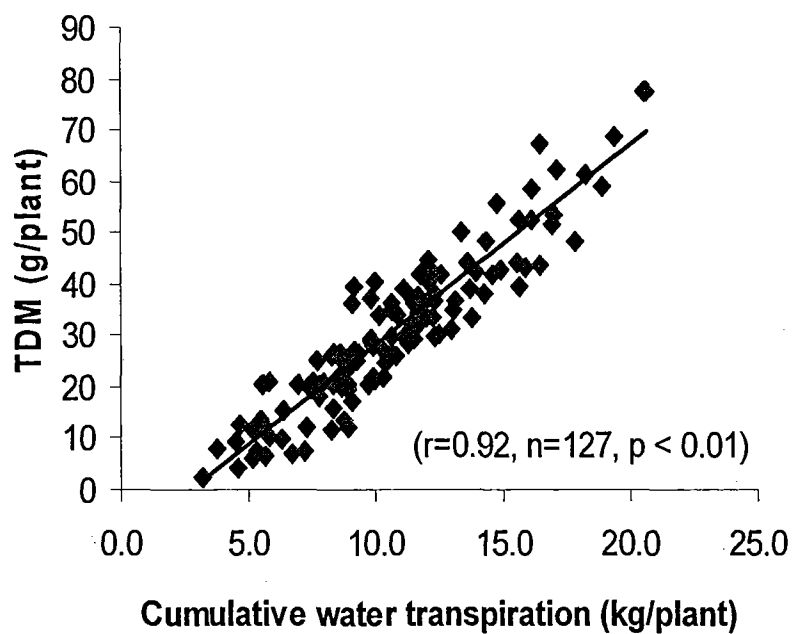


Figure 20: Relationship between cumulative water transpiration and total dry matter in the F1 mapping population (DR-14 x Madagascar) of *C. canephora*.

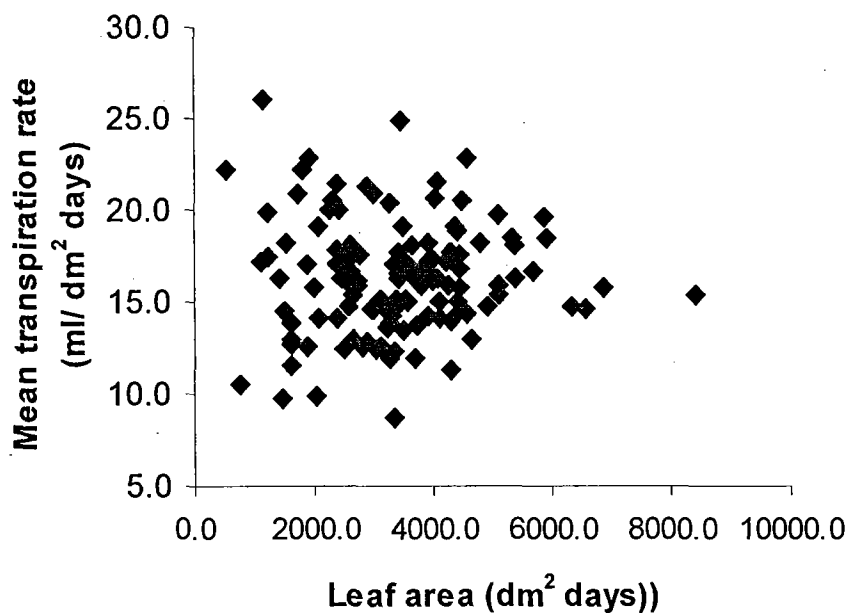


Figure 21: Relationship between leaf area and mean transpiration rate in the mapping population (DR-14 x Madagascar) of *C. canephora* accessions

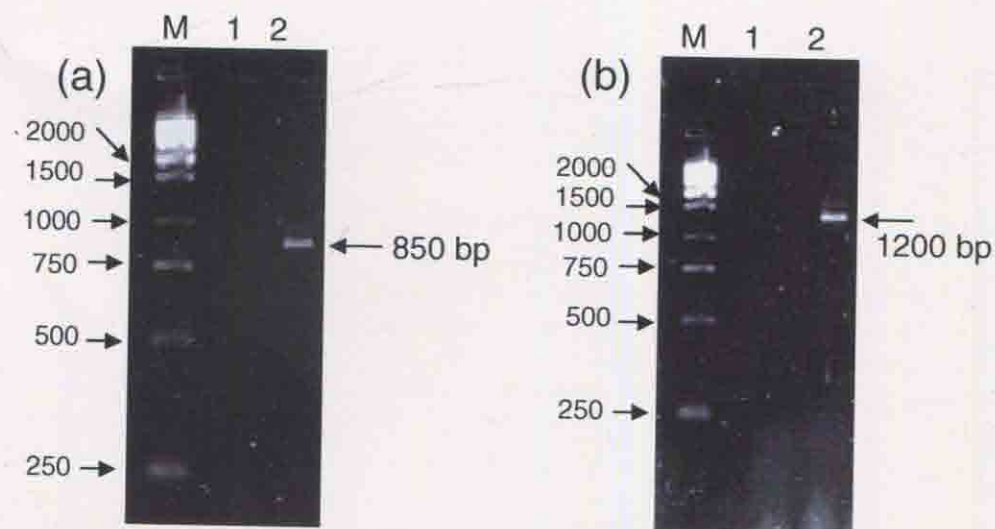


Plate 15. SCAR marker amplification. The developed SCAR markers for root length and root dry weight were amplified with two parental accession having contrasting root length and root dry weight characteristics. (a) shows an amplification of SCARL1 marker in low root length parent, L1 valley (lane 2) and no amplification in high root length parent, S.3334 (lane 1). (b) shows amplification of SCARD1 marker in low root dry weight parent, L1 valley (lane 2) and no amplification in high root dry weight parent, S.3334 (lane 1).



Plate 16: SCARL1 amplification pattern in the low and high root length individuals of mapping population (L1 valley x S.334).

Amongst 134 F1 individuals, the nine individuals with low (lane 1 to 9) and high (lane 10 to 18) root length were selected to evaluate the locus specific SCARL1 marker associated for low root length was amplified with an annealing temperature of 52°C. The position of the marker weight was indicated by arrow; M-1kb marker. The marker was amplified in both the extreme root length individuals indicating the poor association with trait.

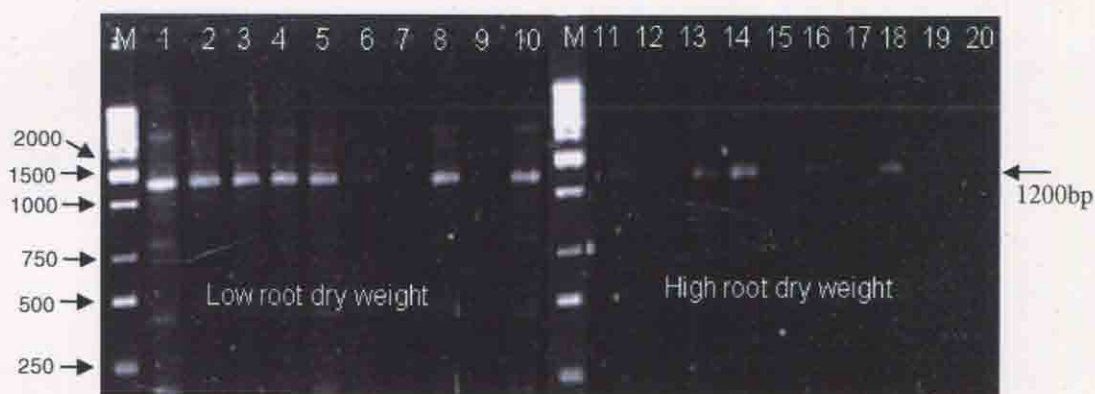


Plate 17: SCARD1 amplification pattern in the low and high root length individuals of mapping population (L1 valley x S.334).

Amongst 134 F1 individuals, the ten individuals with low (lane 1 to 10) and ten high (lane 11 to 20) root dry weight types were selected to evaluate the locus specific SCARD1 marker associated for low root dry weight with an annealing temperature of 59°C. The position of the marker weight was indicated by arrow; M-1kb marker.

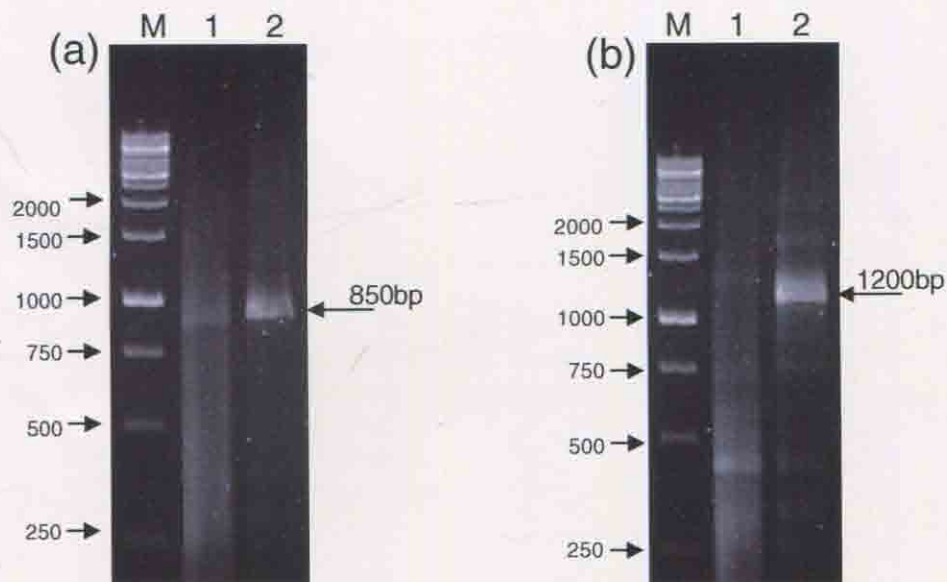


Plate 18. SCAR marker validation. The SCAR markers developed for low root length and low root dry weight were validated in two contrasting accessions with root length and root dry weight characteristics, selected through gravimetric approach. (a), amplification of SCAR1 marker in high root length clone, Madagascar (lane 1) and low root length clone, DR-14 (lane-2). (b), SCAR2 marker amplification in high root dry weight clone, Madagascar (lane 1) and low root dry weight cloen, DR-14 (lane 2). M, 1Kb standard marker

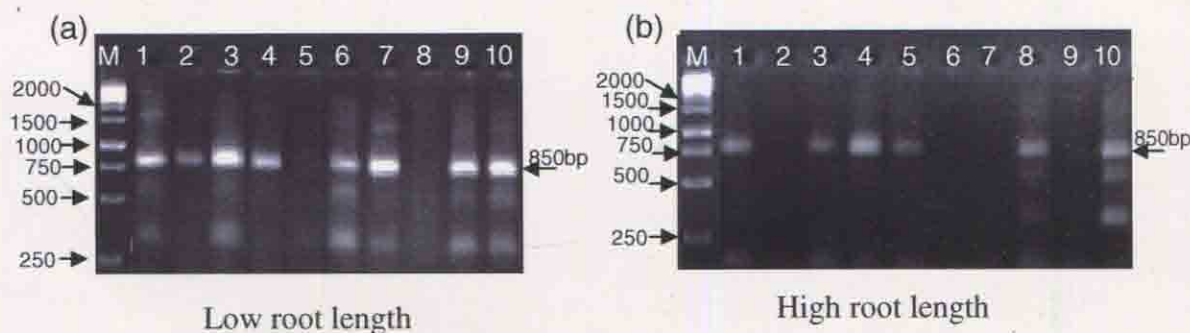


Plate 19. SCARL1 amplification pattern in the low and high root length individuals of mapping population (L1 valley x S.3334).

Amongst 127 F1 individuals, the ten individuals with low (a) and high (b) root length were selected to evaluate the locus specific SCARL1 marker associated for low root length was amplified with an annealing temperature of 52°C. The position of the marker weight was indicated by arrow; M-1kb marker. The marker was amplified in both the extreme root length individuals indicating the poor association with trait.

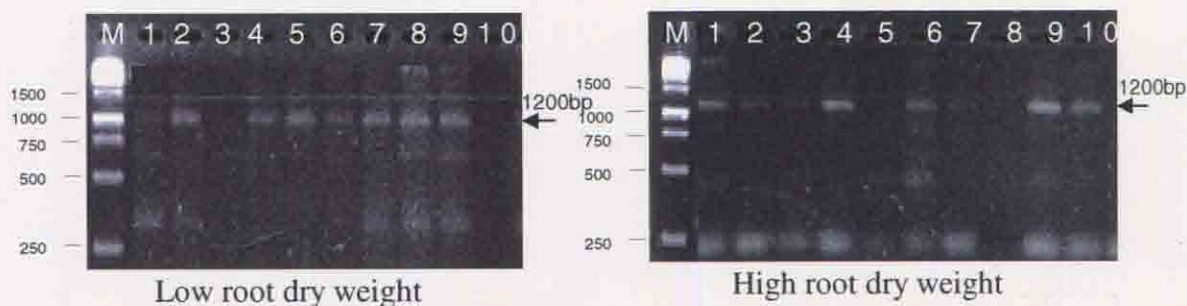


Plate 20. SCARD1 amplification pattern in the low and high root length individuals of mapping population (L1 valley x S.3334).

Amongst 127 F1 individuals, the ten individuals with low (a) and ten high (b) root dry weight types were selected to evaluate the locus specific SCARD1 marker associated for low root dry weight with an annealing temperature of 59°C. The position of the marker weight was indicated by arrow; M-1kb marker.

Discussion

V DISCUSSION

Drought is an environmental factor that produces water deficit or water stress in plants. Internal water deficit in plants is initiated when water potential decreases and cell turgor begins to fall below its maximum value (Kozlowski and Pallardy, 1997). Efforts to increase water harvesting and its utilization efficiency has been the main focus of interest in the recent years. Dehydration avoidance is one of the adoption mechanisms, through which plants survive and yield better under stress conditions. Therefore deep root system is known to be one of the best strategies to cope with the stressful situations (Ingram et al., 1994). In fact enhanced water uptake by efficient root characteristics is a desirable physiological mechanisms of dehydration avoidance under limiting conditions. Hence, identifying genotypes with desirable root characteristics for moisture stress condition would be very useful. (Kramer and Boyer, 1995; Turner, 1997). However knowledge of root traits associated with drought tolerance is important for further understanding drought tolerance mechanisms of the whole plant.

Furthermore, particularly in the tropics, drought episodes are coupled with both high solar radiation and temperature remarkably aggravated (DaMatta, 2003) the water stress. Therefore, thorough analysis of the physiological events during drought stress and their genetic control is essential to define regulatory genes, which are primary and positively contributing to stress tolerance. Coffee is an important plantation crop, grown predominantly in the peninsular India. Growth and productivity of this crop is constrained by the water availability particularly during rain free periods (November to March). In coffee, some physiological traits have been shown to potentially contribute to yield under drought conditions (DaMatta, 2004b), but the development of an efficient breeding method for drought tolerance is still a long-standing objective. However the differential adaptation to drought among arabica and robusta coffee genotypes appears to be behavioural, and predominantly governed by rates of water use or efficiency of extraction of soil water (Meinzer et al., 1990b; DaMatta et al., 2003; Pinheiro, 2004). Therefore, physiological and morphological characteristics, such as, root depth and WUE, should be recommended as potential traits for selecting coffee genotypes with superior performance under drought conditions. In the last 15 years, relatively

considerable amount of basic research on coffee physiology focusing on water relations and drought tolerance mechanisms has been undertaken, but much less research efforts has been devoted to identifying responses and mechanisms of tolerance to unfavourable temperatures.

Though several agronomic interventions to conserve the soil moisture and enhance the water use efficiency (WUE) are advocated, however tailoring varieties to drought tolerant and efficient in water use offers the best long term and cost effective solution to encounter the uncertainty of monsoon and shrinking availability of irrigation water. It is now believed that more rapid progress of the physiological basis of trait performance such as specific leaf area (SLA), ability of root system to capture water for transpiration (T) and partitioning efficiency (p) or harvest index (HI) under drought conditions would bring in selection of high water use efficiency types in coffee (Da Matta and Ramalho, 2006). Therefore, screening and selection of genotypes, which use available water more efficiently and produce more biomass and yield per unit amount of water utilized, is a useful approach for increasing productivity under limited water environments (Passioura, 1986). Thus WUE is considered as an important component of adoption to drought and hence has a relevance both under irrigated and rainfed conditions.

Although impressive progress has been achieved through conventional breeding (Heisey and Edmeades, 1999), the potential of genetic improvement for coffee production under drought conditions is still large. In view of the time-consuming process in coffee breeding, modern molecular technologies like DNA markers have a great potential to assist in and accelerate breeding programmes. Various DNA marker technologies have been applied to coffee DNA fingerprinting; Randomly amplified polymorphic DNA (RAPD); Lashermes *et al.*, 1993; restriction fragment length polymorphism (RFLP); Lashermes *et al.*, 1996a; amplified fragment length polymorphism (AFLP); Pearl *et al.* 2004. To overcome the reproducibility problem associated with the RAPD technique, RAPD markers have been converted into sequence-characterized amplified regions (SCAR; Paran and Michelmore, 1993). SCAR markers have been developed for several crops, although molecular genotyping through SCAR primers has been documented in a few woody species, e.g. olive (Hernandez *et al.*, 2001). These markers can also be potentially used for trait validation studies.

Apart from these markers development of simple sequence repeat (SSR)(Rovelli et al., 2000) are highly polymorphic repeated sequences are informative molecular markers, because they are co-dominant and enable the heterozygous samples to be distinguished from the homozygous. Due to these characteristics SSRs are powerful tools for following specific genes in assisted cross programmes. Such genetic markers gave the ability to track the inheritance of a particular marker allele and a measured quantitative trait provides evidence that linked to QTL is linked to marker.

Therefore molecular marker linkage maps are being used successfully in associating the traits. During the last two decades, using linkage-based association analysis (including QTL interval mapping) a large number of genes for various traits (quality traits, resistance to biotic and abiotic stresses, etc.) has already been tagged with markers (Varshney *et al.*, 2005; Gupta *et al.*, 1999). These associated markers, which can be particularly useful to follow the inheritance of the marker region of the genome. Such markers have been developed for many plant species including in arabica coffee (Pearl *et al.*, 2004) where this genetic map served as the framework for mapping the QTL controlling source-sink traits in the population. Therefore these identified QTLs controlling source sink traits identify markers for selection of coffee quality and productivity. Where as in *Coffea canephora* genetic linkage map was constructed (Lashermes et al., 2001; Hendre *et al.*, 2007), however there is a need of developing a QTL map for the drought-associated traits are very much essential to introgress the traits in the agronomically superior variety.

The results of the present investigation on Development of SSR markers and identification & validation of markers linked to root and associated physiological traits in *Coffea canephora* have been discussed in this chapter.

I. Development of microsatellite markers and their characterization in coffee species

Isolation of simple sequence repeats from *Coffea canephora*

In this study, 23 coffee microsatellites have been isolated using enrichment method. The results have demonstrated the essential need to make use of all tools at hand in attempts to develop a larger number of markers, if marker assisted breeding is to be realized.

Various lessons learnt from this work, form the basis for future research and are therefore discussed in detail in this chapter.

When developing the microsatellite-enriched library for coffee, 500 colonies were screened, 150 recombinant clones ranging from 500-1000bp were selected for further sequencing. Sequence analysis revealed that only 78 clones (52%) contained microsatellite motif regions. while, many traditional microsatellite isolation studies have reported an average of only 2.3% final positive clones (Zane *et al* 2002) after sequencing, the average percent of false positives in such studies has been estimated at 48.7% (Squirrel *et al* 2003). The short length of oligonucleotides used as probes could be a possible reason for the resulting high proportion of false positives. The length of the probe applied for hybridization can influence the type and length of microsatellites captured. Armour *et al* (1994) found that longer oligonucleotide probes not only favoured the isolation of relatively long arrays, but also eliminated the mismatches. While the protocol in this study made use of oligonucleotides 28-30 bp long, other more successful studies (Stajner *et al*, 2005) used much longer probes (200-550 bp). It has been suggested that short probes for hybridization tend to select fragments with shorter interrupted repeats. In other investigations (Lopes *et al.*, 2002; Senda *et al.*, 2004), 2 rounds of hybridization have been performed to eliminate false positives. A second hybridization is especially recommended, if inefficient labeling of oligonucleotide probes (by using incorrect concentration of probes, any form of contamination, or inadequate hybridization and wash steps) was suspected in the first round of screening. In pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Budak *et al.*, 2003) for example, a second round of hybridization managed to discard more than 50% of clones (46 out of 80) as false positives.

Several authors (Edwards *et al.*, 1996; Panaud *et al.*, 1996) have suggested enrichment of genomic libraries to enhance identification of microsatellites. Enrichment ratios published in the literature vary from 20-95% depending mainly on the procedure and method used (Stajner *et al.*, 2005). In fact, the enriched library prepared in this study resulted in a higher percentage (52%) of microsatellites.

Characteristics of isolated SSR markers

In the present study, mono, di, tri and tetra nucleotide repeats were identified in library. Among mono repeats, the motif G repeat was present more than 15 times followed by A and T repeats (Table 3) in the sequences analyzed. In 150 sequenced clones, abundant G and T repeats were identified, but the repeat number was not more than ten nucleotides. We have designed two primers for the G repeats amplification. As such there were no reports on mononucleotide repeats in coffee genome.

In plant genome, abundance of dinucleotide repeats AG and GA class of repeats accounted about 10.5% and 15% respectively in the total SSR identified (Table 3). This result is consistent with those of previous studies in *Coffea* genus (Poncet *et al.*, 2006), hop (*Humulus lupulus* L.) (Stajner *et al.*, 2005), white clover (*Trifolium repens* L.) (Kölliker *et al.*, 2001) and *Lolium temulentum* (Senda *et al.*, 2004), for all of which enrichment strategy was employed. Although database surveys have shown that GA motifs are relatively rare in plants than AG motifs (Wang *et al.*, 1994), the high success of their isolation after enrichment in these studies may suggest that they occur at a significant frequency in the genomes of most plants. The AG, TG, and CA class of repeats have been reported as the most abundant di-nucleotide repeats in coffee genome (Moncada *et al.*, 2004; Rovelli 2000), suggesting the AG and GA repeat could be the most abundant di-nucleotide repeats in most plant genomes. No GC repeats were isolated, and is consistent with the reports that these repeats are extremely rare in most genomes (Cui *et al.*, 2005). Lower frequencies of GC repeats have been attributed to methylation of cytosine, which in turn increases the chance of mutation to thymine, by deamination (Schorderet and Gartlar, 1992).

There are few tri-nucleotide repeats AAC and GAT identified in the enrichment library (Table 4). AAC has been reported to be the most common motif in plant nuclear DNA sequences (Wang *et al.*, 1994). The identified 10 tri-nucleotide repeats were not abundantly noticed in the enrichment library. Wang *et al.*, (1994) found that 57% of the tri-nucleotide repeats motifs in plants contain G-C base pairs within coding regions. Indeed, tri repeats containing the G-C base pairs were noticed in our study, indicating repeats resided in coding regions. A previous study in *Coffea arabica* inbred line (Maluf *et al.*, 2005) reported high proportion of ATC repeats. This is one of the identified repeat

in the present study. The results of this study are therefore consistent with those of other investigators, who reported the most frequent tri-nucleotide repeat motifs in plants

Despite being the most abundant mono and di-nucleotide repeats, the 8 tetranucleotide repeats were also isolated and of which 4 repeats were used for primer designing and amplification. The enrichment protocol was less successful, yielding only 6 positive clones. Gaitán-Solis *et al.*, (2002) suggested that this failure could be due to selective enrichment of specific microsatellite sequences. However, the high success in enrichment of similar motifs in a separate study using similar enrichment (Métais *et al.*, 2002) strategy invalidates this point.

Although di-nucleotide repeats are the most commonly used class of microsatellite markers in plants, there has been concern over the difficulty experienced in genotyping them due to a high frequency of strand slippage artifacts (Levinson and Gutman 1987). In contrast, tri- and tetra-nucleotide repeat based markers have been shown to produce a higher proportion of discrete PCR products due to reduced level of stuttering (Gastier *et al.*, 1995). In human, these motifs have been found in genes that are associated with several neurological diseases such as the X syndrome (Jin and Warren 2000), Huntingdon's disease and several forms of ataxia (Sermon *et al.*, 2001) and myotonic dystrophy (Timchenko *et al.*, 2001). These disorders are caused by excessive expansions of triplet repeats located near or within coding regions (Fujimori *et al.*, 2003).

The sequencing was done in both the directions in order to increase the quality of sequences for primer design after alignment. About 49% of all primers designed were amplified with efforts on optimization. The correct PCR conditions were established through optimization. Although Squirrel *et al.*, (2003) have indicated that optimization in itself could be a source of attrition and other investigations have pointed out the fact that PCR optimization may lead to insignificant improvement in band interpretability (Ashworth *et al.*, 2004).

Cross amplification of microsatellite primers

A high level of polymorphic loci was noted whatever the species considered. Although the conserved nature of EST-SSR promoted transferability than genomic SSRs and it

could also have limited polymorphism. This has been suggested in several reports where the level of EST–SSR polymorphism was lower than that with SSR derived from genomic libraries (Cho *et al.*, 2000; Eujayl *et al.*, 2001; Gupta *et al.*, 2003). However, some recent studies reported high levels of polymorphism with EST–SSR markers (Eujayl *et al.*, 2004; Fraser *et al.*, 2004; Saha *et al.*, 2004), with cases where EST–SSR markers were associated with equivalent or even higher levels of polymorphism than genomic SSR (e.g. Liewlaksaneeyanawin *et al.*, 2004; Varshney *et al.*, 2005a). In our study the developed genomic SSRs showed a good amount of transferability in nine *Coffea* species also supported these observations.

Here we demonstrated that primer sets derived from cultivated *C. canephora* were validated not only in the related diploid cultivated species of *C. canephora* but also in other distant related species, as previously suggested by Combes *et al.* (2000) using 11 SSR primers. The cross amplification was obtained in several crops such as tomato (Alvarez *et al.*, 2001), as well as on trees (Dirlewanger *et al.*, 2002 on *Prunus*; Marques *et al.*, 2002 on *Eucalyptus*, Poncet *et al.* 2004 on *Coffea* species). Indeed, tomato primer sets generated amplification products not only in the *Lycopersicon* genus but also in potato and pepper (Alvarez *et al.*, 2001) and microsatellites developed from *Eucalyptus grandis* and *E. urophylla* amplified orthologous loci in *E. tereticornis* and *E. globulus* (Marques *et al.*, 2002).

In this study, twelve primers showed allelic length variation with less monomorphism and eight primers failed with the amplification for particular locus in nine species. The absence of alleles at particular locus is called null allele. This shows potential cause for the occurrence of microsatellite with null allele in the absence of primer sequences in the targeted genomic region and hence genomic region could have caused the absence of the primer annealing sites of these microsatellite primers resulting in the null alleles as reported in the other alien gene transferred wheat lines (Malik *et al.*, 2003; Adhikari *et al.*, 2004; Vikal *et al.*, 2004). This indicates that most of the species may distantly relate to the cultivated species, and this was noticed in cassava (Roa *et al.*, 2000).

2. Molecular analysis of *Coffea canephora* mapping population by using identified polymorphic RAPD and SSR markers.

Segregation of RAPD markers in Segregating F1 mapping population

One of the main uses of DNA markers in agricultural research has been the construction of linkage maps for diverse crop species. Linkage maps have been utilized for identifying chromosomal regions that contain genes controlling simple traits (controlled by single gene) and quantitative trait using QTL analysis (reviewed by Mohan *et al.*, 1997). The process of construction of linkage maps and conducting QTL analysis to identify genomic regions associated with traits is known as QTL mapping (also 'genetic,' 'gene' or 'genome' mapping) (McCouch and Doerge, 1995; Mohan *et al.*, 1997; Paterson, 1996a,b). DNA markers that are tightly linked to agronomically important genes (called gene 'tagging') may be used as molecular tools for marker assisted selection (MAS) in plant breeding (Ribaut & Hoisington, 1998). MAS involves using the presence/absence of a marker as a substitute for or to assist in phenotypic selection, in a way which may make it more efficient, effective, reliable and cost effective compared to conventional plant breeding methodology. Several DNA marker systems like RAPD, SSR, ISSR, AFLP, etc., and associated techniques are available today for fingerprinting the plant germplasm and analyzing the segregation of traits in the mapping population, but information on their relative usefulness in particular crops is limited.

In the present investigation, an attempt was made to assess the segregation of RAPD and SSR markers in the F1 generation. The RAPD markers are of dominant in nature (Williams *et al.*, 1990) and these markers are efficiently used to study the segregation of dominant traits in the mapping population. However the SSR markers are being co-dominant in nature also plays a significant role in mapping QTLs regulating morpho-physiological traits. These markers were used in many of the crops (Michelmore *et al.*, 1991; Ohmori *et al.*, 1995), and also in the coffee genome (Pearl *et al.*, 2004; Lashermers *et al.*, 2001). In the previous study, 31 *C. canephora* accessions were gravimetrically characterized for root traits and two contrasting parents were selected, having high root type (S.3334) and low root type (L1 valley) (Awati, 2004). These selected contrasting ones were crossed, obtained 134 mapping individuals were phenotypically characterized for the root & other associated physiological traits and

genotypically characterized with RAPD markers. In the present study further genotyping was carried with more RAPD and SSR marker system.

To study the segregating characters in the F1 generation, the parental accessions were screened with 300 RAPD (OPK1 to OPZ20) and 55 SSR primers. Only 41 RAPD and 9 SSR primers showed polymorphism between the parents. These RAPD and SSR primers generated 70 & 9 polymorphic loci respectively (Table 3). This revealed the low level of genetic variation between two contrasting parents. So knowledge of the amount of genetic variability in a crop species is very important for any breeding programme, since it provides the basis for the development of new genotypes. The low level of DNA polymorphism between the two parental accessions, coupled with the large number of common bands, implies the genomes of the sample under study are rather homogeneous. Therefore any polymorphic marker is important within this narrow genetic base. Similar results have been noticed in *Arachis hypogea* (Halward *et al.*, 1992) and *Allium cepa* (Wilkie *et al.*, 1993). The reason for this differential rate of variation in RAPD pattern at the intraspecific level is unknown (Pillay & Kenny 1996). The obtained polymorphic markers were characterized in the F1 progeny for the segregation analysis (plate 8a to 8f and plate 9a & 9b).

The studies, analyzing the segregation of RAPDs (Echt *et al.*, 1992; Roy *et al.*, 1992) have put-forth two assumptions concerning these markers: (1) each band is considered to represent a single locus and is (2) a dominant marker for that locus. On this basis, the presence of a fragment in a parent is considered to be either in the homozygous dominant or the heterozygous condition. It is not possible to distinguish homozygosity from heterozygosity at a locus from the presence of a RAPD band (Carlson *et al.*, 1991). A parent not containing a fragment is assigned the recessive genotype and containing a fragment is known as dominant. When a marker is present in one parent and absent in the other ($Aa \times aa$ or $aa \times Aa$) the marker will segregate in a 1:1 ratio in the F1 generation. When a marker is present in both parents ($Aa \times Aa$), the expected segregation ratio for that marker in the progeny is 3: 1. We have analyzed our data within the framework of these assumptions. All the RAPD markers in the F1 generation of *C. canephora* (Table 9.1) segregated in ratios that were not consistent with Mendelian inheritance. Chi-square tests were performed for each of the markers to determine if segregation differed from the

expected allele frequency ratio of 1:1 ratios. At a significance level of $P = 0.05$ all the tests, about 62% of the marker loci were in agreement with the expected ratios and 38% of the marker loci not followed the Mendelian inheritance. It is expected that RAPD bands of maternal DNA origin would show non-Mendelian inheritance. However there is no suitable explanation for the nuclear genome. RAPD markers that do not follow basic genetic models may not be useful in breeding (Pillay and Kenny, 1996).

This study has, however, provided the basis for such research. Polymorphic RAPD markers in parental plants segregated as independent alleles in the F1 progeny of *C. canephora* suggesting that RAPDs have a genetic basis and may be useful in linkage mapping. In fact the nine-microsatellite markers did not follow the Mendelian segregation in the F1 progeny, which could be due to parental heterozygous characteristics.

Single marker analysis for root and associated physiological traits

Complex physiological traits have on recent occasions been described by a small number of major QTL (Kearsey and Farquhar, 1998). Problems arise in finding useful QTL for a particular trait when there are numerous QTL associated with it, as the smaller their individual contribution, the more difficult they are to detect. Indeed Single-marker analysis is generally a good choice when the goal is simply detection of a marker linked to a trait. However, estimation of its position and its effects requires further complex analysis such as marker regression (Kearsey and Hyne, 1994) or interval analysis (Haley and Knott, 1992).

In the present investigation, study was to associate molecular markers with the trait of our interest, in particular to identify the markers linked to locus controlling root traits. It has been well documented that unlike arabica (Bhat, 2002), root spread and penetration is relatively poor in robusta. Hence, any morphological, physiological and genetic markers to identify root traits have considerable importance.

However Single Marker ANOVA was initially used to identify markers showing significant association with traits (Table 3.1) (using SAS software with $P \leq 0.05$). The extent of association of marker and variation explained by the markers for the morpho-physiological traits are presented in Table 5,6,7,8 & 9.

Despite these efforts, no successful linkage map could be obtained, partly due to the inadequate number of polymorphic markers generated from this study. The output of the association analysis indicates that, out of 29 traits, only 24 traits were linked to 85 markers. Even markers linked to traits explaining the phenotypic variation was not more than 10%. In the present study, phenotypic variation explanation for all the traits ranges from 2.61- 9.86%. This suggests that the successful application of molecular markers in trait mapping is greatly requires more polymorphic markers. This could be achieved through ISSR, SNPs, SSRs and AFLP markers system. While successful mapping attempts could still be carried out in the absence of a linkage map as done in chickpea (Chandra *et al.*, 2004).

Most of the markers explained phenotypic variance between 3-4%, however only two markers, i.e., OPZ11₁₅₀₀ and OPV14₅₀₀ were significantly ($p < 0.01$) linked to photosynthetic rate (Pn), accounting the variation of about 9.23% and 9.30% respectively (Table 16). The root traits such as root length, number of secondary roots, root dry weight and root: shoot ratio, revealed significant association ($p < 0.05$) with markers and the phenotypic variance ranges from 2.95% to 6.86% (Table 13). This suggests the trait variance explanation is not sufficient to answer the probabilities of occurrence of these markers. However, the root length and root: shoot ratio was linked with five markers each and even these markers have explained not more than 5% phenotypic variance. This demonstrates that the ability of the marker analysis has not revealed cryptic markers that linked to root traits and also one could hypothesize that there are limited number of polymorphic markers. The phenotypic traits like number of leaves, stem dry weight and root dry weight are associated with only one marker. This indicates that the insufficient markers could not associate with the traits. Further saturation with polymorphic markers is greatly required for association study.

The single-marker ANOVA analysis shows significant association of more than five markers for each trait (overlapping markers), such as root length, root: shoot ratio, WUE, $\Delta^{13}\text{C}$ and photosynthesis (Pn) (Table 18). This signifies the quantitative nature of the traits (Fig. 4) and hence many genes control these traits. In fact our results demonstrate that the RAPD markers such as OPK11_{1000bp}, OPY20_{1200bp}, OPZ10₁₃₅₀ and other markers were associated with more than one trait. This highlights one of the problems of marker

trait analysis in single-marker ANOVA, because we cannot conclude the additive and multiplicative effect of each marker for the particular trait by single marker analysis. Another serious limitation is that a locus with major effect and weak association cannot be distinguished. This could be solved by using more number of marker system and developing the QTL interval map (Lander and Botstein, 1989), thus permitting detection of QTL in each interval (described as a bin) lying between any two flanking markers that individually may show association with the trait.

In the present study, co-dominant SSR markers were also used for the marker-trait association study. The SSR markers identified herein are significantly associated with morphological and physiological traits, but these markers were explaining the trait in the range between 2.83% to 7.19% (Table 17). However, not much literature on SSR marker usage in constructing the linkage map and association study is available in coffee. Although construction of linkage map was done in *C. arabica* (Pearl, 2004) and *C. canephora* (Lashermes, 2001) predominantly using AFLP and RAPD markers, but association with the traits were not attempted. However the first molecular linkage map generated using pseudo-testcross strategy for *Coffea canephora* (CxR x Kagnalla), has the largest number of mapped SSRs (71 genomic, including nine EST-SSRs) (Hendre *et al.*, 2007). Since these markers were locus specific and if they associated for specific traits, they could be used to validate the marker in other germplasm/accessions. One such study was reported in rice (Chaitra *et al.*, 2006), where they validated the RM201 marker linked to root length in rice diverse accessions to identify the low and high root length accessions.

3. Development of locus specific sequence characterized amplified region (SCAR) marker from RAPD markers linked with root trait.

The utility of RAPDs can be enhanced by manipulation of primer sequence to produce desired amplification for genotyping (Dinesh *et al.*, 1993), and the conversion of RAPDs to SCAR is one such method. The SCAR-PCR is known to palliate some of the drawbacks of RAPD-PCR (Paran and Michelmore, 1993). The SCAR markers are also advantageous as they identify a single or few bands instead of a complex pattern (Agusti *et al.*, 1999). Therefore RAPDs can be transformed into SCARs (Sequence Characterized

Amplified Regions) by sequencing the polymorphic DNA fragments and synthesizing two primers homologous to each end (Paran and Michelmore 1993). Conversion into SCAR markers is usually considered desirable before application in marker assisted breeding, since the process improves significantly the reproducibility and reliability of PCR assays (Herna'ndez *et al.*, 1999; Chowdhury *et al.*, 2001; Zhang and Stommel 2001).

In this work, SCAR markers have been successfully developed for two traits, such as root length and root dry weight. The polymorphic RAPD band, which was amplified by a specific primer, was cloned and directly sequenced (Plate 11). Using sequence information the two SCAR primers (SCARL1 & SCARD1) were developed and amplified in specific accessions to get the required length of the band.

Our result demonstrated the amplification of single specific required fragment in the accession of specific character and it is comparable to the specific RAPD amplified fragment. This indicates the reproducibility of amplification, because SCAR primer will amplify the specific locus. Hence SCAR markers could be of used in the validation study, provided it is developed with background of specific trait association.

4. Validation of SCAR markers in the mapping population of *Coffea canephora* (L1 valley x S.3334)

Markers should also be validated by testing for their presence on a range of cultivars or genotypes or in the trait specific mapping population (Sharp *et al.*, 2001). Even when a single gene controls a particular trait, there is no guarantee that the DNA markers identified in one population will be useful in different populations, especially when the populations originate from distantly related germplasm (Yu *et al.*, 2000) or population developed with the same trait backgrounds. In the present study based on single marker analysis, the RAPD markers associated with low root length and root dry weight were converted into SCAR markers. To reconfirm the statistical association of markers with the traits, the developed SCAR markers for the low root length designated as (SCARL1) and for low root dry weight, named as SCARD1 were validated in the parental plants (L1 valley x S.3334). The amplification was positive at corresponding size of the polymorphic RAPD band for both the traits in the low root length and low root dry

weight parent, L1 valley, (Plate 15). This corroborates the amplification of primers at a particular annealing temperature and also the marker associated with the trait.

Amongst phenotypically characterized 134 F1 individuals, obtained by crossing L1 valley x S.3334, ten representing the high and low root length were identified for validating the low root length associated SCARL1 marker (Table 32). The amplification was noticed in seven out of nine plants in both the low and high root length individuals (Plate 16). This shows that the marker was not tightly associated with the trait. Similarly ten F1 progenies representing the low and high root dry weight individuals were identified for validating the SCARD1 marker. In this case also we could notice amplification in both the low and high root dry weight types, however the low root dry weight types showed 80% of the amplification compare to high root dry weight with only 40% (Plate 17). This further signifies that more number of markers is essential to find an exact region of the DNA segment for a particular trait.

Assessment of genetic variability in root traits, WUE and associated physiological traits under different moisture regimes

To revalidate the developed SCAR marker for the trait of interest in another set of mapping population, contrasting parents for root traits were selected and crossed. The parental plants were selected based on the phenotypic and genotypic observations.

Genetic variability in roots

To identify and breed for a deep root system to exploit as much of water from the soil is necessary under water deficit conditions. Under such situations identification of genetic variability in the root traits is important for the selection of better root type. In this context, comparative studies in 35 *C. canephora* accessions, both under 60% FC and 80% FC conditions evidenced the identification of better rooting types. The result demonstrates lot of genetic variability among the accessions, but the mean root dry weight between two moisture stresses was not significant. However the root dry weight under stress condition was comparatively maintained as that of non-stress condition. Similar reports are available on this aspect, where in, it has been shown that absolute root biomass can be maintained, or in certain cases, it may be even higher under stress conditions (Volkmar, 1997). It is suggested that such a thing can occur under moisture

stress conditions, because severe stress inhibits both root and shoot growth (Sharp and Davies, 1979). Our result demonstrates that the maintenance of root dry weight under moisture stress conditions is an adoptive strategy of dehydration postponement mechanism (Nunes, 1976; Meinzer *et al.*, 1990b; DaMatta *et al.*, 2000, 2003; Pinheiro *et al.*, 2005; Praxedes *et al.*, 2005b). The reported genetic variability in the root traits and the relevance of this trait as an important avoidance strategy has been well documented in the literature which justifies that breeding for better root characteristics would be rewarding.

Since coffee is a shade loving plant and strongly sensitive to water deficit. Consequently the high relative water content (RWC) maintained by coffee leaves under dehydrating conditions (Nunes, 1976, Da matta *et al.*, 1993, 2002a) appear to be a means of drought avoidance, rather than tolerance. Such a high RWC has been associated with conservation of a high symplast volume, which appears to be more directly related to maintenance of root growth under water deficit conditions. Hence, deeper root system would favourably maintains the internal water status, by tapping the water from deeper soil layer and thus maintaining more RWC in the leaves. The observed significant relationship between the root dry weight v/s total transpiration (CWT) and mean transpiration at both the moisture regimes further signifies the importance of roots.

These 35 *C. canephora* accessions once again showed consistent root characteristics when they were maintained under 80% FC in the second experiment. A strong positive correlation was observed between the root dry weights under stress (60% FC) v/s non-stress (80% FC), stress v/s non-stress in experiment-2 and vice versa (Fig. 10a, b, c). This signifies that the intrinsic character of the accessions in maintaining root biomass at all levels of moisture stress and it also indicates that the root dry weight is independent of stress experienced by the plant (low G x E interaction). This could be a good selective trait for the breeding programme.

In contrast, the shoot biomass was significantly reduced as root biomass increased; this intern showed a significant difference in root shoot ratio (RSR) between the treatments. The total reduction of RSR over the stress (60% FC) was 27%. This kind of variations in the root and shoot biomass really varies the total biomass. This demonstrates under dry conditions the relative allocation of biomass to roots usually increases (Hamblin *et al.*,

1991). The benefit of higher allocation of biomass to roots is an increased capacity for water uptake. The costs of a larger root system are the cost of construction (possibly at the expense of construction of photosynthetic tissue) and the increased respiratory demand associated with its maintenance. So it can be hypothesized that greater allocation of biomass to roots is associated with benefits in terms of water uptake capacity with costs in terms of carbon. It could be due to similar reasons that in the present study the root to shoot ratio increased at 60% FC. This differential response of root to shoot growth at lower water potentials is advantageous and could be a means by which plants avoid excess dehydration.

Water use efficiency (WUE)

WUE is another important physiological trait, which has significance both under stress and non-stress conditions. Generally, WUE increases under moderate moisture stress conditions (Foarquhar *et al.*, 1989; Condon *et al.*, 1990; Udyakumar *et al.*, 1998). Hence identifying genotypes with high WUE and high biomass under moisture stress condition would be rewarding. In fact at the same time it may not be so easy to have such genotypes, because of the interrelationships with other physiological characteristics. For example, better stomatal control of transpiration under stress results in increase in WUE under stress (Condon *et al.*, 1990; Richards *et al.*, 1993), but results in low growth rates due to impaired carbon exchange rates, resulting in decrease in photosynthesis.

The physiological processes that regulate WUE have been fairly elucidated. In view of its importance the genetic variability and the heritability characteristics for WUE have been extensively studied in the recent years (Wright *et al.*, 1994; Ray *et al.*, 1999). The reported genetic variability in WUE and high broad sense heritability makes it a relevant trait for crop improvement. So far much less attention has been paid in studying or identifying the genotypes with high root traits and WUE at the productivity level. This trait has more relevance in perennial plant species especially when there is no option for further water mining.

In the present study, a significant variation in WUE was noticed between the treatment and within the accessions. However WUE under moisture stress condition was increased to the extent of 25% compared to control condition. This suggests under stress conditions

the accessions could use the available water efficiently by reducing transpiration rate. This transpiration efficiency is regulated by stomatal factors (g_s) and mesophyll efficiency (g_m) and perhaps this provides an option to identify the better accessions, which are having moderate WUE with high RSR.

A positive and significant relationship between CWT and root dry weight indicates that more the root growth more is the water transpired. This is true in the genotypes having high RSR by virtue of which they transpire more water and thereby down the WUE. However, an inverse relationship between mean transpiration rate (MTR) and WUE further indicates that *C. canephora* could be a conductance type, which is predominantly brought about by stomatal factors. This greatly corroborates with the result of 31 accessions studied for the root and other associated physiological traits (Awati, 2004). Most of the plants have evolved to maximize WUE through reduction in transpiration linked with g_s (Udayakaumar *et al.*, 1998). Since dry matter production is strongly associated with total transpiration and reduction in transpiration results in low crop growth rates. Keeping all these views, using standardized normal distribution, we found that some accession like Madagascar, Saigon, Ivorycost and DR-8 maintained more TDM, RSR and moderate WUE (Fig 26 & 27) under both the levels of moisture stresses and even these accessions existed consistently in the second experiment. This suggests the existing genetic variability would facilitate to identify and exploit traits for introgression in crop breeding. These accessions could also be used as better rootstocks for high yielding clones.

However under stress condition the leaf area duration (LAD) and net assimilation rate (NAR) were positively correlated to CWT, which indicates that assimilation increases as transpiration rate and duration of functional leaf area increases. The fact that NAR was not related to WUE under both the levels of moisture stress indicates low stomatal resistance and mesophyll characteristics like carboxylation efficiency and carbon exchange ratios are not contributing for the variation in WUE.

There are possible deviations from these generalizations, supported by the evidence generated in this study. Further confirmation of the consistency of the genotypes with regard to deviation would be necessary and interesting.

Genetic diversity analysis of *C. canephora* using RAPD markers

The assessment of diversity based on agro-morphological parameters have been often hampered by the lack of adequate data on distinguishing morphological characters and confusions due to the wide variations for a particular phenotypic trait in a given population. However, DNA marker technologies, presently dominated by RAPD, AFLP, ISSR and SSR or microsatellites largely overcome these limitations (Karp *et al.*, 1997).

The associations among genotypes were estimated using UPGMA-clustering analysis for both genotypic and morphological data. The accessions were properly clustered according to physiological and agronomic features. Though we have used exotic accessions of different geographical origin with local collected accessions, they clustered based on plant characteristics than geographical origin. According to Lashermes *et al.*, (1999) grouping on the dendrogram in *C. arabica* collected from different countries were based on geographical origins. Hence our result demonstrates accessions relevant to their geographical origin were not showed.

The genetic variability of 35 accessions of *C. robusta* was evaluated using a combination of the Random Amplified Polymorphic DNA (RAPD) technique. The genetic variability and the relatedness among all accessions were initially evaluated using 17 informative RAPD primers rendered 454 bands, of which, 143 (31.5%) were polymorphic, which revealed a very low level of genetic variation.

In the present study we have assessed the structure of genetic diversity in 10 exotic and 25 indigenous *C. canephora* accessions and also the pattern of diversity in comparison with morphological data. The collected 10 exotic robusta (Java to Ivory cost) seedlings brought from respective countries & collections were established at Central Coffee Research Institute, Balehonnur. However DR1 to DR20 were collected during the severe drought season of 1983, Regional Coffee Research Station, Chundale, where botanists conducted a survey and identified some good plants from various estates of Wayanad. They collected the open pollinated seeds from these plants and named as drought resistant (DR) collections from 1-20. The other accessions like Sib-4, Sib-7, Robusta bold, Robusta high yield and dwarf C x R were also estate collections identified from private estates of Wayanad.

From the data, it is apparent that the robusta accessions did not tend to show good amount of genetic diversity. The dendrogram constructed based on Ward's method of clustering revealed a maximum linkage distance of 80 units for RAPD markers and 5500 units for morphological characters for the 35 *C. canephora* accessions. Though they are different in total linkage distance, however number of clusters formed and some of the accessions belonging to each cluster were fairly same. It indicates the expression of phenotypic characters independent of environmental interaction and they are genetically more stable.

Based on genetic distance the clustering of accessions demonstrates that the exotic coffee accessions did not shown much genetic diversity among them, except the Uganda 880 grouped with DR collections. In fact the considerable genetic diversity was detected in drought resistance (DR) collections. On the other hand five private estate collections such as Sib-4, Sib-7, Robusta bold, Robusta high yield and dwarf CxR were made into a separate group with some DR collections. This demonstrates the genetic base was narrower among the exotic accessions compare to DR collections (Masumbuko *et al.*, 2003). Since the genetic variation in so many cultivars is generally believed to be more the result of spontaneous mutations of major genes and increased possibility of combining different alleles results in higher heterozygosity (Carvalho 1988, Fontes *et al.*, 2000). Hence our result could corroborate with the above said statement.

A relatively close relationship was revealed among the exotic accessions, which clustered together with the values of genetic dissimilarities ranging from 11 to 12%, except Uganda 880 with 17%. In the DR collections, lot of dissimilarity was observed ranging from 9 to 31%. However the least dissimilarity was observed in private estate collections. This examination of the genetic diversity was consistent with dissimilarity values. The dissimilarity matrix based on SED revealed a maximum of 23- 31% dissimilarity between all 35 accessions. Except this accession in all other accessions not much dissimilarity was observed. Based on principal component analysis RAPD markers and physiological parameters showed relatively low genetic diversity. This indicates, since *Coffea* germplasm collections were established in many countries throughout the world, it has been reported that genetic base of cultivated varieties is rather narrow (Berthaud and

Charrier, 1988). Under this circumstance, one would expect a reduced variability even at DNA level. Nevertheless, the molecular markers were able to distinguish among all 35 accession used in this study.

Mapping population

In annual crops the parental lines, which are generally homozygous, are crossed to raise the F₂ mapping population. However in perennials the germplasm lines and the cultivated accessions are often highly heterozygous and hence construction of an appropriate mapping population is the main problem, because of its long vegetative period and limited fruits/nuts production within a fixed time period. The heterozygosity is thus associated with greater adaptation and hence production in these clones is not uniform. From this context, F₁ population developed from heterozygous parents show considerable phenotypic and genetic variations. In apple, conifers and coconut, full sib and half sib family was used as mapping population by considering it as a pseudo test cross (Weeden, 1994; Bandaranayake and Kearsey, 2006). Similar approach has been adapted to cross the contrasting parents in the present investigation to develop mapping population.

Based on genetic linkage distance (dendrogram) and physiological traits in 35 accessions of *C. canephora*, contrasts were identified (Fig. 12) with respect to root to shoot ratio and total dry matter (DR-14 and Madagascar). Further they were crossed and 127 F₁ mapping individuals were produced to study the segregation of root traits.

The drawback for assessing variability in physiological and morphological traits is lack of replications, since each individual in the mapping population is different from one other. When the trait is influenced by environmental factors phenotyping of mapping population will be extremely difficult. From this context, it is desirable to quantify a specific trait by direct and indirect methods. For instance, in the present investigation physiological trait like WUE was estimated gravimetrically by measuring the TDM and total transpiration.

One of the objectives of studying the mapping population was to score the variability in roots and other physiological traits. The results demonstrated significant variability and quantitative nature of the traits (Table. 30 & Fig. 71). This suggests that these traits are controlled by multiple genes, each segregating according to Mendel's laws and the

degrees to which the varying environment can also affect these traits. In spite of continuous variation in these traits, we could find some relations among the physiological traits in the mapping population (Table 31).

Root biomass is under genetic control with relatively high heritability under well watered and drought conditions (Ehdaie *et al.*, 2001). Though there were huge variations in root dry weight (2.87-22.45 g/plant), the correlation was positive with TDM and CWT (Fig. 18, 20). This shows that stomatal conductance, transpiration and assimilation rate was mainly dependent on root system and therefore when the supply of water from the roots is inadequate, stomata closes, photosynthesis shuts down, and plants can wilt. Hence root growth determines the above ground biomass. In addition to this variation in several physiological traits such as leaf area, LAD, NAR and MTR were observed. In the F1 mapping individuals, a negative relationship between MTR and WUE (-0.27) confirmed that robusta coffee is a conductance type. A positive significant relation between leaf area and shoot dry weight determines that the photosynthesizing area could certainly increase the above ground biomass. However the relationship between leaf area and MTR signifies that some transgressive segregants could be identified in the population.

Validation of SCAR markers in F1 mapping individuals (DR-14 x Madagascar)

The SCAR markers developed from the L1 valley x S.3334 mapping population for the root traits were validated in the mapping population (DR-14 x Madagascar), which was developed on the same background. Initially the parental plants, DR.14 having low root length & low root dry weight and Madagascar with high root length & high root dry weight was selected among 35 accessions by gravimetric basis. Using these parental DNA, the SCARL1 and SCARD1 was amplified, and was found positive for both the markers in low root length and low root dry weight parent (Plate 18). This indicates that the markers are associated for the traits. These parents were crossed and obtained 127 F1 mapping individuals. These were phenotypically characterized for the root and other associated physiological characteristics gravimetrically. From the obtained population ten representative individuals were selected with low & high root length and root dry weight for the SCAR marker validation (Table 33). However the low root length associated SCARL1 failed to show the association with the trait, because it amplified in both low

and high root length individuals (Plate 19). Similarly, SCARD1 also showed its amplification in both root dry weight type individuals (plate 20). However the amplification pattern corroborates, previously selected representative mapping individuals (L1valley x S.3334). This further confirms that, the associated markers are not significantly linked to the traits. Suppose these markers followed the Mendellian inheritance, it could fail to show the expected phenotypic variance.

Out come

From our study we have developed twenty-three microsatellite markers (SSRs) from the genomic DNA of elite variety (S.3334) of *Coffea canephora*. The developed SSR marker were validated by amplifying in nine *Coffea* species, and also used in parental polymorphic studies. Among newly developed 23 SSR primers, two were (CPCM 11 and 13) showed a polymorphism and characterized in the mapping population.

From the single marker analysis, the marker statistically associated for root length (OPS_{1850bp}) and root dry weight (OPL1_{1450bp}) were converted into SCAR markers and validated in the two phenotypically characterized mapping population. Due to low phenotypic variance explained by these markers the successful validation was not achieved for both the traits.

Summary

VI. SUMMARY

Root system and stress-induced drought avoidance and resistant strategies form important components of drought resistance. A dynamic root system is fine-tuned to soil moisture status and is known to regulate the amount of water available to the plant depending on its distribution in the soil. Despite ample genetic variation for many root related parameters, genetic improvement of root characteristics in coffee using conventional selection has been difficult and progress towards improvement of yield in rainfed has been hampered. A significant proportion of the phenotypic variability of several of these putative drought resistance traits is explained by the segregation of relatively few genetic loci, thus leading to the possibility of indirect selection of these complex traits by means of marker-assisted selection (MAS) strategy. Considering these limitations to efficient selection, molecular marker technology is a powerful tool for selecting such traits. The major emphasis in this investigation has been development of genomic SSRs, identification and validation of molecular markers associated for root traits in the mapping population. The results of these experiments are summarized in this chapter.

6.1 Development of microsatellite markers

Highly informative molecular markers, such as simple sequence repeats (SSRs), can greatly accelerate breeding programs. The aim of this study was to develop and characterize a comprehensive set of SSR markers for coffee (*Coffea canephora*), which can be used to tag genes and quantitative trait loci controlling traits of agronomic interest. Sequence analysis of 150 clones from genomic libraries enriched for (CA)₁₇, (AG)₁₆, (AGC)₈, (AGG)₈, (AAC)₁₄, (ATC)₁₄, (AAG)₁₄, (ACGC)₅, and (ACCT)₈ repeats yielded 78 clones containing SSR loci. The majority of SSRs consisted of perfect dinucleotide repeats, only 7% being trinucleotide repeats. After exclusion of SSR loci with less than 15 bp sequences, 55 potentially useful SSRs remained. However due to insufficient flanking regions and non-compatibility of primer pairs, 23 SSR loci were designed and PCR products in the expected size range were amplified.

Successful cross-species amplification was achieved in nine coffee tree species for 20 of the 23 primer pairs. These markers were tested in parental polymorphism (L1 valley x

S.3334) to characterize the allelic segregation in an F_1 population. However, only two of the twenty primer pairs (CPCM 11 & CPCM 13) showed polymorphism.

6.2 Identification of polymorphic RAPD and SSR markers and study of segregation of polymorphic loci in mapping population (L1 valley x S.3334)

In the previous study, contrasting parents such as L1 valley having low root type and S.3334 with high root type were selected among 31 accessions characterized for root traits by gravimetric approach. These parents were crossed, and derived 134 mapping individuals were phenotypically characterized for root and other associated physiological traits. The contrast parental plants were genotyped with 21 RAPD polymorphic primers and generated 42 polymorphic loci were molecular characterized in 134 mapping individuals.

In the present study, the existing parental plants were genotyped with 20 RAPD and 9 SSR polymorphic primers and these were respectively generated 28 and 9 informative loci. The generated informative 79 loci were molecular characterized in the mapping progeny, to study the segregation of parental alleles in the 134 mapping individuals. The Chi-square test was performed for each of the markers to determine the observed frequencies of presence versus absence of the fragment corresponded to the expected 1:1 F_1 ratio from crossing $aa \times Aa$. Of 70 RAPD polymorphic loci, 42 followed the expected ratio (1:1), in which only 7 markers were significant at $P=0.001$, 3 of them were significant at $P=0.05$ and rest of 32 marker loci were significant at $P=0.95$. This study has, however, provided the basis for polymorphic RAPD markers in parental plants segregated as independent alleles in the F_1 progeny of *Coffea canephora* suggesting that RAPDs have a genetic basis and may be useful in linkage mapping. However the 9-microsatellite primers have not followed the Mendelian inheritance.

6.2.1 Association of markers with trait

In the single marker analysis, simple linear regression model was examined to study the association between marker loci (independent) & trait score (dependent variable). Of the 32 phenotypic traits, 29 traits were associated with 42 markers (56%) at significance level of 5%. The phenotypic variance (R^2) explained by each trait ranged from 2.61-9.73%. However 30% of the markers explained the trait variance range of 3-4%. There are 13

putative RAPD markers associated for root traits such as root length, no. of secondary roots, root dry weight and root to shoot ratio, in which root length associated marker OPS1₈₅₀bp showed high phenotypic variance of 6.86%. However, only two microsatellite markers linked to root length (CPCM13₄₀₀) and root to shoot ratio (CM211₃₀₀). In our study it was found that 25 markers associated with more than one trait and few of the markers associated with positively related traits such as OPP5₁₈₀₀ (LAD & shoot dry weight), OPZ10₁₀₃₀ (MTR & g_s) and OPK20₃₅₀ (Ci & NAR).

6.3 Development and validation of SCAR markers in the mapping population of *Coffea canephora* (L1 valley x S.3334)

To enable trait-specific selection, two RAPD markers associated for root length (OPS1₈₅₀) and root dry weight (OPL1₁₂₀₀) were successfully converted into sequence characterized amplified region (SCAR) markers and designated as SCARL1 and SCARD1 respectively. To confirm the statistical association with the markers and the traits, the developed SCAR makers were validated in the same mapping population (L1 valley x S.3334). Of the 134 mapping individuals, ten representative individuals with low and high root length progenies were selected (Table 32), and amplified with SCARL1. The amplification showed in 7 low root length types and 4 high root length types (Plate 16), whereas SCARD1 showed amplification was also followed the same range of amplification in low and high root dry weight types (Plate 17). To revalidate these markers, another trait specific mapping population was developed.

6.3.1 Development of trait specific mapping population

To develop a trait specific mapping population, 35 accessions were phenotyped for root and associated physiological traits, in two separate pot culture experiments by gravimetric approach. The salient findings have been summarized below.

- A significant genetic variability was noticed among 35 accessions for root traits like root length, root dry matter, root to shoot ratio and also in WUE.
- In this study significant positive correlation was recorded between root dry weight under stress and non-stress conditions, indicating low G x E interaction.

- Strong positive correlation between root dry weight and total dry matter under two moisture regimes ($r= 0.95$) signifies the importance of root trait.
- Strong positive associations between root dry weight and cumulative water transpired ($r= 0.87$) signifies the importance of root system to mine the water for sustainable growth.
- Negative correlation between WUE and MTR in both the experiments suggests the stomatal regulation of WUE ($r= -0.59$).

6.3.2 Study of genetic diversity of Coffee accessions

Thirty-five accessions were genotyped with 17 polymorphic and reproducible primers and generated 454 bands were employed in STASTICA programme. The Ward's method of cluster analysis grouped these accessions into three major clusters and clustering was based on low, medium and high root to shoot ratio types. Genetic relatedness among these accessions was evaluated by generating dissimilarity matrix. It is revealed that the low dissimilarity among the accessions, except DR-8, DR-3, DR-9 and DR-4. Further principal component analysis revealed tight clustering of these accessions in three dimensions, indicating relative low molecular diversity. However the genetic distance was also calculated for phenotypic traits and compared with phenotypic data. This indicates the phenotypic traits and genetic characters are positively related and this would be desirable for the breeding strategy. Based on Z distribution analysis (Fig 12), Madagascar having good root system and DR-14 having poor root system were crossed and 127 mapping individuals were developed.

6.3.3 Phenotyping of the mapping population

Phenotyping for the root and other associated physiological traits in the mapping population was carried through gravimetric basis. The assessed traits showed continuous distribution indicating the quantitative nature (Fig 17). As it was observed in the different accessions, even mapping population showed strong positive association of root dry weight with shoot dry weight, total dry matter and cumulative water transpired (Fig 18). Therefore, the present study indicates that the root parameters play a major role in sustaining growth and productivity in water limited conditions for coffee.

6.3.4 Validation of SCAR markers validation in DR14 x Madagascar mapping population

In the marker assisted selection, it is important to validate their association with the specific traits. Of the 127 mapping individuals, ten representative low and high root length sample individuals were amplified with SCARL1. Out of ten individuals, eight low root length and six high root length individuals showed amplification. Similarly, SCARD1 associated for root dry weight showed in seven low root dry weight types and five high root dry weight types in ten representative individuals. This indicates that regardless of the significance level of the marker, may not tightly linked/associated with the trait. Nevertheless these markers revealed an association; a tight association was not evident. Further experiments are underway with more markers. Hence further saturating with more markers would certainly help in identifying tightly linked markers for the traits.

Conclusions

1. Twenty locus specific genomic SSR markers were developed and validated in the *Coffea* species.
2. Markers associated for the trait of interest are converted into SCAR markers and validated in another trait specific mapping population

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Appendix

APPENDICES - II

Phenotypic data of *Coffea canephora* mapping population (DR-14 x Madagascar)

Plant No.	Plant height (cm)	No. of nodes	No. of primaries	No. of leaves	Stem Girth	Root length (cm)	Leaf dry weight (g)	Stem dry weight (g)	Shoot dry weight	Root dry weight
1	57.00	33.00	8.00	65.00	10.02	64.00	27.64	16.64	44.28	15.80
2	65.00	25.00	5.00	48.00	9.50	56.00	20.04	15.93	35.97	9.55
3	66.00	24.00	8.00	48.00	11.82	46.00	23.43	16.70	40.13	13.60
4	66.00	23.00	6.00	46.00	11.90	46.00	25.24	17.49	42.73	11.74
5	64.00	25.00	6.00	49.00	10.22	55.00	20.42	13.96	34.38	11.72
6	62.00	41.00	3.00	28.00	8.94	51.00	14.40	11.30	25.70	8.16
7	64.00	22.00	6.00	43.00	11.96	54.00	22.22	20.69	42.91	12.08
8	60.00	29.00	8.00	56.00	10.52	51.00	25.02	17.82	42.84	10.68
9	61.00	16.00	4.00	32.00	10.86	49.00	22.15	15.71	37.86	10.86
10	61.00	29.00	8.00	56.00	11.62	41.00	30.14	23.48	53.62	17.31
11	66.00	32.00	8.00	60.00	11.70	49.00	37.74	21.47	59.21	13.71
12	59.00	22.00	6.00	42.00	9.80	42.00	25.43	15.90	41.33	13.39
13	45.00	22.00	6.00	43.00	9.62	56.00	21.47	12.22	33.69	12.91
14	56.00	23.00	5.00	45.00	11.64	67.00	18.33	17.48	35.81	15.04
15	77.00	30.00	7.00	58.00	10.68	65.00	24.70	22.92	47.62	16.60
16	71.00	37.00	9.00	74.00	112.64	60.00	41.91	33.21	75.12	14.07
17	63.00	35.00	9.00	77.00	11.80	35.00	19.69	14.68	34.37	9.61
18	61.00	29.00	8.00	58.00	11.00	66.00	39.87	23.18	63.05	16.18
19	60.00	25.00	7.00	49.00	9.28	41.00	27.37	17.08	44.45	12.01
20	64.00	23.00	4.00	44.00	10.36	67.00	21.03	15.53	36.56	11.33
21	65.00	29.00	8.00	57.00	10.82	57.00	24.61	17.53	42.14	11.88
22	64.00	15.00	3.00	27.00	9.96	52.00	19.99	13.08	33.07	8.73
23	46.00	22.00	6.00	43.00	8.92	49.00	14.92	10.72	25.64	6.35
24	64.00	20.00	5.00	39.00	8.68	36.00	17.79	12.27	30.06	6.67
25	46.00	19.00	5.00	36.00	8.40	55.00	11.44	7.77	19.21	6.27
26	76.00	26.00	8.00	51.00	10.90	56.00	23.79	16.11	39.90	8.53
27	62.00	20.00	4.00	40.00	8.94	37.00	17.78	13.54	31.32	8.48
28	53.00	26.00	7.00	49.00	9.42	58.00	22.01	18.01	40.02	11.42
29	74.00	25.00	8.00	52.00	9.86	49.00	24.17	14.15	38.32	11.62
30	48.00	13.00	3.00	25.00	7.52	38.00	11.06	7.14	18.20	5.70
31	60.00	19.00	6.00	38.00	10.30	46.00	18.75	12.46	31.21	9.51
32	60.00	23.00	6.00	45.00	10.64	54.00	19.28	12.45	31.73	9.41
33	62.00	18.00	4.00	35.00	10.98	38.00	15.98	13.10	29.08	8.78
34	65.00	19.00	6.00	37.00	9.34	53.00	19.43	18.32	37.75	8.90
35	54.00	21.00	5.00	40.00	8.08	46.00	13.78	11.00	24.78	7.54
36	54.00	22.00	6.00	43.00	8.64	39.00	14.57	10.69	25.26	7.09
37	57.00	21.00	4.00	41.00	8.40	68.00	18.70	11.07	29.77	8.56
38	45.00	20.00	5.00	39.00	7.32	52.00	10.53	7.42	17.95	5.57
40	55.00	22.00	6.00	43.00	9.00	50.00	17.66	12.50	30.16	6.61
41	73.00	13.00	1.00	25.00	10.96	40.00	12.24	13.14	25.38	6.97
42	60.00	25.00	6.00	49.00	10.92	38.00	27.12	17.15	44.27	10.35
43	55.00	12.00	2.00	24.00	8.66	53.00	11.60	10.05	21.65	5.74
44	68.00	33.00	9.00	65.00	11.32	65.00	29.34	21.10	50.44	14.05
45	79.00	27.00	10.00	54.00	11.28	43.00	27.52	24.14	51.66	13.51
46	64.00	40.00	10.00	74.00	10.40	55.00	28.54	19.40	47.94	13.91

Plant No.	Plant height (cm)	No. of nodes	No. of primaries	No. of leaves	Stem Girth	Root length (cm)	Leaf dry weight (g)	Stem dry weight (g)	Shoot dry weight (g)	Root dry weight (g)
47	71.00	30.00	7.00	55.00	12.06	47.00	28.58	31.71	60.29	13.73
49	64.00	31.00	9.00	62.00	12.22	54.00	32.99	28.55	61.54	19.09
50	59.00	15.00	4.00	30.00	9.48	42.00	15.33	11.81	27.14	8.50
51	76.00	31.00	8.00	60.00	12.68	50.00	36.75	30.31	67.06	22.42
52	44.00	17.00	0.00	32.00	5.66	44.00	11.83	8.67	20.50	3.87
53	66.00	19.00	5.00	38.00	11.04	51.00	22.06	19.89	41.95	13.48
54	54.00	14.00	4.00	28.00	8.88	56.00	11.78	9.58	21.36	6.31
55	60.00	19.00	3.00	36.00	8.62	64.00	15.70	12.41	28.11	8.31
56	62.00	17.00	4.00	34.00	9.10	39.00	18.66	11.54	30.20	8.27
57	60.00	14.00	3.00	28.00	10.86	41.00	16.07	12.70	28.77	9.51
58	64.00	11.00	0.00	20.00	9.14	40.00	19.34	8.46	27.80	5.08
59	65.00	24.00	8.00	47.00	11.32	75.00	18.68	15.97	34.65	10.66
61	38.00	8.00	0.00	16.00	8.04	51.00	7.82	4.66	12.48	6.95
62	64.00	25.00	7.00	47.00	9.86	39.00	14.89	14.70	29.59	6.73
63	52.00	19.00	4.00	38.00	9.84	62.00	14.99	11.52	26.51	6.92
64	69.00	17.00	5.00	34.00	10.90	59.00	17.15	18.77	35.92	9.96
65	46.00	14.00	1.00	28.00	7.08	44.00	8.52	5.46	13.98	3.84
66	59.00	14.00	2.00	28.00	9.44	44.00	14.36	12.85	27.21	6.60
67	59.00	17.00	4.00	34.00	8.36	69.00	12.36	9.69	22.05	6.82
69	59.00	13.00	2.00	26.00	9.66	54.00	10.08	7.36	17.44	6.30
70	43.00	14.00	2.00	28.00	7.38	50.00	9.75	5.33	15.08	4.54
72	70.00	19.00	4.00	37.00	9.58	52.00	18.24	12.65	30.89	9.50
73	38.00	11.00	2.00	22.00	7.20	31.00	7.36	3.19	10.55	3.55
74	68.00	29.00	8.00	58.00	9.18	47.00	29.28	16.16	45.44	5.87
75	74.00	17.00	5.00	33.00	9.28	46.00	17.55	15.74	33.29	8.23
76	66.00	17.00	4.00	33.00	8.22	61.00	14.99	12.08	27.07	5.28
77	62.00	28.00	8.00	56.00	9.06	56.00	25.94	18.65	44.59	11.01
78	54.00	29.00	8.00	56.00	9.72	63.00	22.77	16.32	39.09	9.54
79	62.00	18.00	4.00	33.00	7.98	59.00	4.89	10.60	15.49	6.00
80	55.00	21.00	6.00	42.00	10.86	40.00	19.57	17.33	36.90	8.78
81	68.00	25.00	6.00	50.00	11.00	71.00	27.69	22.53	50.22	13.37
82	72.00	26.00	8.00	52.00	11.18	74.00	26.18	21.36	47.54	12.75
83	55.00	16.00	4.00	32.00	11.04	38.00	15.86	14.15	30.01	7.01
84	68.00	22.00	6.00	42.00	11.40	58.00	22.13	20.61	42.74	11.27
85	66.00	19.00	3.00	36.00	10.76	55.00	18.70	17.35	36.05	12.13
86	72.00	22.00	6.00	44.00	9.38	58.00	23.19	16.73	39.92	12.11
87	76.00	13.00	2.00	24.00	8.74	38.00	14.20	11.20	25.40	7.45
88	58.00	20.00	4.00	40.00	8.66	54.00	18.43	12.79	31.22	7.46
89	51.00	17.00	2.00	34.00	10.10	51.00	14.75	10.80	25.55	7.16
90	47.00	15.00	3.00	30.00	7.74	49.00	11.72	7.42	19.14	6.01
91	54.00	17.00	4.00	32.00	8.88	58.00	13.30	10.14	23.44	6.41
92	71.00	19.00	6.00	38.00	10.54	41.00	22.83	17.68	40.51	8.96
93	44.00	11.00	2.00	22.00	6.88	61.00	7.15	6.17	13.32	5.40
94	73.00	25.00	8.00	49.00	9.72	52.00	26.62	17.65	44.27	11.50
95	57.00	19.00	5.00	36.00	8.14	46.00	14.60	9.83	24.43	6.94

Plant No.	Plant height (cm)	No. of nodes	No. of primaries	No. of leaves	Stem Girth	Root length (cm)	Leaf dry weight (g)	Stem dry weight (g)	Shoot dry weight (g)	Root dry weight (g)
96	59.00	15.00	4.00	30.00	8.98	64.00	12.49	11.74	24.23	8.07
98	83.00	29.00	8.00	57.00	9.18	41.00	25.75	20.89	46.64	9.43
99	65.00	15.00	4.00	30.00	8.40	55.00	16.21	13.77	29.98	8.51
100	46.00	23.00	6.00	43.00	8.32	50.00	15.01	11.09	26.10	7.81
101	56.00	23.00	6.00	45.00	9.78	57.00	19.96	13.87	33.83	10.21
102	46.00	11.00	2.00	21.00	7.46	39.00	9.41	6.45	15.86	5.30
104	58.00	15.00	3.00	28.00	8.46	48.00	15.20	10.21	25.41	7.01
105	58.00	22.00	6.00	43.00	9.00	35.00	25.35	16.65	42.00	10.49
106	50.00	22.00	6.00	44.00	8.86	44.00	19.95	10.20	30.15	7.76
107	60.00	21.00	6.00	38.00	9.28	57.00	18.19	14.01	32.20	9.94
108	72.00	20.00	4.00	40.00	10.02	52.00	21.21	19.59	40.80	8.29
109	67.00	23.00	6.00	34.00	9.38	49.00	23.25	16.33	39.58	10.18
110	64.00	23.00	5.00	45.00	11.82	59.00	25.52	14.60	40.12	10.90
111	61.00	20.00	6.00	39.00	9.62	65.00	22.09	15.92	38.01	11.78
112	73.00	22.00	6.00	43.00	11.68	69.00	31.66	24.31	55.97	14.27
113	72.00	21.00	5.00	42.00	9.74	48.00	22.13	17.35	39.48	8.13
114	64.00	19.00	4.00	37.00	8.40	58.00	18.78	13.31	32.09	9.10
115	73.00	18.00	5.00	35.00	9.32	60.00	20.12	18.21	38.33	9.78
116	74.00	24.00	7.00	46.00	10.44	49.00	29.81	21.16	50.97	10.92
117	57.00	10.00	0.00	20.00	8.34	36.00	7.99	6.35	14.34	4.11
118	70.00	25.00	7.00	48.00	10.42	53.00	23.84	19.33	43.17	10.41
119	75.00	20.00	6.00	40.00	10.88	45.00	18.53	15.04	33.57	7.05
120	63.00	15.00	3.00	29.00	9.16	56.00	13.38	12.29	25.67	6.71
121	54.00	22.00	6.00	46.00	9.92	58.00	23.11	12.77	35.88	10.09
122	53.00	16.00	3.00	31.00	8.40	49.00	15.38	9.68	25.06	7.30
123	75.00	15.00	3.00	29.00	9.62	60.00	16.72	13.43	30.15	9.33
124	54.00	10.00	0.00	19.00	7.76	47.00	9.34	8.60	17.94	6.58
125	51.00	21.00	5.00	41.00	8.74	52.00	18.93	15.62	34.55	7.02
126	46.00	12.00	3.00	22.00	7.58	43.00	9.75	6.86	16.61	5.45
127	52.00	17.00	2.00	34.00	7.72	47.00	15.03	10.38	25.41	6.10
128	62.00	28.00	7.00	55.00	10.56	70.00	34.07	21.55	55.62	11.83
129	51.00	21.00	8.00	46.00	8.54	56.00	19.84	16.34	36.18	9.03
130	62.00	23.00	4.00	45.00	9.48	48.00	18.61	15.53	34.14	8.78
131	31.00	17.00	5.00	34.00	5.82	70.00	9.91	5.81	15.72	3.41
132	49.00	13.00	4.00	25.00	7.34	52.00	10.32	7.31	17.63	2.87
133	56.00	18.00	3.00	35.00	8.26	75.00	11.32	8.57	19.89	3.62
137	45.00	15.00	6.00	30.00	7.32	57.00	16.37	10.68	27.05	5.35

Plant No.	RSR	TDM	Delta TDM	CWT	WUE	LA-1	LA-2	LAD	NAR	MTR
1	0.36	60.08	48.18	17.91	2.69	775.29	5984.52	9091.94	5.30	1.97
2	0.27	45.52	33.62	12.05	2.79	484.01	4754.49	7045.78	4.77	1.71
3	0.34	53.73	41.83	14.63	2.86	476.19	6215.91	9000.87	4.65	1.63
4	0.27	54.47	42.57	11.89	3.58	624.31	5288.46	7952.68	5.35	1.50
5	0.34	46.10	34.20	11.71	2.92	463.58	4835.26	7126.94	4.80	1.64
6	0.32	33.86	21.96	8.47	2.59	453.12	3853.14	5791.92	3.79	1.46
7	0.28	54.99	43.09	15.91	2.71	536.87	4939.92	7366.28	5.85	2.16
8	0.25	53.52	41.62	12.62	3.30	798.95	5125.59	7968.51	5.22	1.58
9	0.29	48.72	36.82	12.31	2.99	466.83	4837.99	7134.98	5.16	1.73
10	0.32	70.93	59.03	18.95	3.12	725.28	5435.81	8286.67	7.12	2.29
11	0.23	72.92	61.02	18.33	3.33	901.13	7722.90	11599.32	5.26	1.58
12	0.32	54.72	42.82	14.95	2.86	581.75	5222.40	7806.58	5.49	1.92
13	0.38	46.60	34.70	10.61	3.27	658.15	4948.92	7541.51	4.60	1.41
14	0.42	50.85	38.95	13.69	2.85	725.47	5316.92	8127.01	4.79	1.68
15	0.35	64.22	52.32	15.69	3.33	758.73	4890.45	7598.15	6.89	2.06
16	0.19	89.19	77.29	20.59	3.75	666.90	9273.08	13369.27	5.78	1.54
17	0.28	43.98	32.08	11.47	2.80	590.07	4419.55	6737.94	4.76	1.70
18	0.26	79.23	67.33	16.53	4.07	945.52	7443.41	11283.11	5.97	1.47
19	0.27	56.46	44.56	12.07	3.69	805.60	4632.36	7314.06	6.09	1.65
20	0.31	47.89	35.99	9.09	3.96	644.62	4355.61	6725.31	5.35	1.35
21	0.28	54.02	42.12	13.95	3.02	738.60	5142.05	7909.47	5.33	1.76
22	0.26	41.80	29.90	11.13	2.69	728.39	5168.94	7931.91	3.77	1.40
23	0.25	31.99	20.09	8.63	2.33	669.28	3493.02	5598.29	3.59	1.54
24	0.22	36.73	24.83	7.69	3.23	652.15	4117.86	6415.66	3.87	1.20
25	0.33	25.48	13.58	8.77	1.55	669.23	3320.76	5366.54	2.53	1.63
26	0.21	48.43	36.53	13.13	2.78	565.44	5097.12	7616.14	4.80	1.72
27	0.27	39.80	27.90	9.85	2.83	960.97	4053.36	6744.27	4.14	1.46
28	0.29	51.44	39.54	15.67	2.52	735.36	5644.46	8580.86	4.61	1.83
29	0.30	49.94	38.04	14.31	2.66	826.35	5167.47	8061.69	4.72	1.78
30	0.31	23.90	12.00	7.29	1.65	604.70	2376.54	4009.77	2.99	1.82
31	0.30	40.72	28.82	11.33	2.54	767.26	4321.15	6843.91	4.21	1.66
32	0.30	41.14	29.24	11.55	2.53	619.59	4655.12	7094.48	4.12	1.63
33	0.30	37.86	25.96	10.81	2.40	519.92	3225.73	5037.90	5.15	2.15
34	0.24	46.65	34.75	13.07	2.66	747.16	4341.46	6844.19	5.08	1.91
35	0.30	32.32	20.42	8.93	2.29	673.11	3228.03	5247.03	3.89	1.70
36	0.28	32.35	20.45	8.69	2.35	606.00	3404.48	5394.10	3.79	1.61
37	0.29	38.33	26.43	8.55	3.09	598.85	4091.11	6308.00	4.19	1.36
38	0.31	23.52	11.62	5.11	2.27	468.94	2477.61	3963.11	2.93	1.29
40	0.22	36.77	24.87	8.80	2.83	657.18	3819.76	6021.48	4.13	1.46
41	0.27	32.35	20.45	7.81	2.62	663.00	2748.78	4588.84	4.46	1.70
42	0.23	54.62	42.72	12.21	3.50	769.20	4863.05	7575.38	5.64	1.61
43	0.27	27.39	15.49	6.37	2.43	452.57	2912.45	4525.95	3.42	1.41
44	0.28	64.49	52.59	16.17	3.25	996.96	7177.74	10994.97	4.78	1.47
45	0.26	65.17	53.27	17.11	3.11	702.65	6200.03	9284.10	5.74	1.84
46	0.29	61.85	49.95	13.39	3.73	969.15	5791.57	9093.17	5.49	1.47

Plant No.	RSR	TDM	Delta TDM	CWT	WUE	LA-1	LA-2	LAD	NAR	MTR
47	0.23	74.02	62.12	17.17	3.62	854.38	5361.27	8360.05	7.43	2.05
49	0.31	80.63	68.73	19.47	3.53	1042.85	6776.73	10517.34	6.53	1.85
50	0.31	35.64	23.74	8.93	2.66	584.82	3585.54	5609.13	4.23	1.59
51	0.33	89.48	77.58	20.69	3.75	1114.47	6731.31	10552.57	7.35	1.96
52	0.19	24.37	12.47	4.65	2.68	612.78	2871.84	4686.81	2.66	0.99
53	0.32	55.43	43.53	16.51	2.64	627.81	4309.65	6640.88	6.55	2.49
54	0.30	27.67	15.77	8.31	1.90	379.18	2576.03	3974.76	3.97	2.09
55	0.30	36.42	24.52	8.71	2.82	581.23	4105.87	6304.15	3.89	1.38
56	0.27	38.47	26.57	8.37	3.17	678.93	4539.58	7018.90	3.79	1.19
57	0.33	38.28	26.38	9.35	2.82	702.04	3598.01	5783.57	4.56	1.62
58	0.18	32.88	20.98	5.83	3.60	640.21	2345.49	4015.77	5.22	1.45
59	0.31	45.31	33.41	13.77	2.43	1070.82	3840.07	6605.15	5.06	2.08
61	0.56	19.43	7.53	7.22	1.04	563.16	2843.62	4582.12	1.64	1.58
62	0.23	36.32	24.42	10.41	2.35	575.63	3291.12	5200.78	4.70	2.00
63	0.26	33.43	21.53	9.95	2.16	657.36	3447.92	5521.60	3.90	1.80
64	0.28	45.88	33.98	10.17	3.34	526.98	3167.98	4969.72	6.84	2.05
65	0.27	17.82	5.92	5.11	1.16	529.01	2461.08	4021.67	1.47	1.27
66	0.24	33.81	21.91	10.33	2.12	594.49	2766.25	4520.20	4.85	2.29
67	0.31	28.87	16.97	9.07	1.87	575.64	3478.57	5452.91	3.11	1.66
69	0.36	23.74	11.84	8.89	1.33	539.18	1998.08	3412.61	3.47	2.61
70	0.30	19.62	7.72	3.77	2.05	549.90	2330.11	3873.61	1.99	0.97
72	0.31	40.39	28.49	11.25	2.53	776.74	4267.43	6784.41	4.20	1.66
73	0.34	14.10	2.20	3.21	0.69	643.32	1615.79	3038.50	0.72	1.06
74	0.13	51.31	39.41	9.13	4.32	866.83	5152.66	8096.21	4.87	1.13
75	0.25	41.52	29.62	10.61	2.79	831.57	4393.70	7027.99	4.21	1.51
76	0.20	32.35	20.45	5.59	3.66	533.64	4212.98	6384.20	3.20	0.88
77	0.25	55.60	43.70	13.69	3.19	809.80	4774.56	7510.96	5.82	1.82
78	0.24	48.63	36.73	11.75	3.13	816.43	4503.06	7154.71	5.13	1.64
79	0.39	21.49	9.59	6.33	1.52	442.12	3358.57	5111.93	1.88	1.24
80	0.24	45.68	33.78	10.87	3.11	665.70	4290.88	6666.60	5.07	1.63
81	0.27	63.59	51.69	16.97	3.05	714.87	6251.50	9369.77	5.52	1.81
82	0.27	60.29	48.39	14.39	3.36	750.49	5323.55	8169.58	5.92	1.76
83	0.23	37.02	25.12	9.23	2.72	537.58	3421.15	5324.49	4.72	1.73
84	0.26	54.01	42.11	12.09	3.48	821.77	4274.76	6854.83	6.14	1.76
85	0.34	48.18	36.28	10.61	3.42	780.40	4754.06	7443.85	4.87	1.43
86	0.30	52.03	40.13	9.99	4.02	943.11	3960.97	6595.99	6.08	1.51
87	0.29	32.85	20.95	7.95	2.64	584.56	3441.21	5414.66	3.87	1.47
88	0.24	38.68	26.78	9.11	2.94	610.87	4156.81	6412.53	4.18	1.42
89	0.28	32.71	20.81	7.55	2.76	646.67	3752.12	5916.37	3.52	1.28
90	0.31	25.15	13.25	5.45	2.43	513.51	2719.29	4348.12	3.05	1.25
91	0.27	29.85	17.95	7.73	2.32	752.91	3886.31	6239.75	2.88	1.24
92	0.22	49.47	37.57	11.71	3.21	832.18	4954.28	7782.79	4.83	1.50
93	0.41	18.72	6.82	6.67	1.02	408.21	2093.20	3364.40	2.03	1.98
94	0.26	55.77	43.87	13.67	3.21	1124.42	5301.41	8642.74	5.08	1.58
95	0.28	31.37	19.47	7.47	2.61	725.62	3221.70	5309.15	3.67	1.41
96	0.33	32.30	20.40	9.71	2.10	465.17	3136.13	4843.75	4.21	2.00

Plant No.	RSR	TDM	Delta TDM	CWT	WUE	LA-1	LA-2	LAD	NAR	MTR
98	0.20	56.07	44.17	15.63	2.83	893.40	5272.75	8293.47	5.33	1.88
99	0.28	38.49	26.59	10.59	2.51	868.22	3613.62	6028.07	4.41	1.76
100	0.30	33.91	22.01	9.91	2.22	884.74	3238.75	5546.09	3.97	1.79
101	0.30	44.04	32.14	11.67	2.75	898.44	4186.92	6839.81	4.70	1.71
102	0.33	21.16	9.26	4.46	2.07	420.12	2446.94	3856.20	2.40	1.16
104	0.28	32.42	20.52	6.97	2.94	506.90	3508.78	5401.09	3.80	1.29
105	0.25	52.49	40.59	12.09	3.36	849.08	5409.77	8418.15	4.82	1.44
106	0.26	37.91	26.01	8.27	3.15	770.72	4201.78	6688.01	3.89	1.24
107	0.31	42.14	30.24	12.49	2.42	648.91	3723.08	5880.33	5.14	2.12
108	0.20	49.09	37.19	9.82	3.79	666.56	4256.41	6621.39	5.62	1.48
109	0.26	49.76	37.86	11.25	3.37	630.20	4641.06	7089.84	5.34	1.59
110	0.27	51.02	39.12	11.09	3.53	882.10	5491.88	8573.00	4.56	1.29
111	0.31	49.79	37.89	12.37	3.06	583.21	4502.01	6839.62	5.54	1.81
112	0.25	70.24	58.34	16.17	3.61	651.97	6538.01	9670.52	6.03	1.67
113	0.21	47.61	35.71	11.81	3.02	454.08	4934.04	7247.02	4.93	1.63
114	0.28	41.19	29.29	9.81	2.99	707.81	4595.43	7132.86	4.11	1.38
115	0.26	48.11	36.21	11.49	3.15	657.59	4429.34	6841.92	5.29	1.68
116	0.21	61.89	49.99	13.43	3.72	499.89	5981.24	8717.12	5.73	1.54
117	0.29	18.45	6.55	5.61	1.17	460.66	1966.79	3264.92	2.01	1.72
118	0.24	53.58	41.68	11.75	3.55	874.90	5240.76	8225.56	5.07	1.43
119	0.21	40.62	28.72	9.81	2.93	936.00	3521.84	5995.79	4.79	1.64
120	0.26	32.38	20.48	7.77	2.64	620.52	3971.85	6176.74	3.32	1.26
121	0.28	45.97	34.07	10.83	3.15	966.85	4410.71	7232.82	4.71	1.50
122	0.29	32.36	20.46	7.59	2.70	830.78	3645.42	6020.49	3.40	1.26
123	0.31	39.48	27.58	10.20	2.70	760.71	4250.90	6740.62	4.09	1.51
124	0.37	24.52	12.62	5.59	2.26	541.71	2465.12	4044.19	3.12	1.38
125	0.20	41.57	29.67	12.33	2.41	867.37	4686.27	7469.65	3.97	1.65
126	0.33	22.06	10.16	5.78	1.76	355.68	2277.54	3541.68	2.87	1.63
127	0.24	31.51	19.61	8.91	2.20	569.22	2909.19	4678.46	4.19	1.90
128	0.21	67.45	55.55	14.75	3.77	944.97	5972.87	9304.49	5.97	1.59
129	0.25	45.21	33.31	12.27	2.71	519.74	4778.84	7126.59	4.67	1.72
130	0.26	42.92	31.02	12.95	2.40	610.06	4119.70	6361.53	4.88	2.04
131	0.22	19.13	7.23	5.29	1.37	169.78	2090.22	3039.70	2.38	1.74
132	0.16	20.50	11.61	8.23	1.41	94.59	2666.73	3713.98	3.13	2.22
133	0.18	23.51	3.95	4.55	0.87	157.20	1371.24	2055.75	1.92	2.21
137	0.20	32.40	20.50	8.37	2.45	107.33	3392.27	4706.96	4.36	1.78