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Preliminary studies on selection indices for activating seedling growth in mangosteen (*Garcinia mangostana* L.)

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Key words: D^2 values, dendrogram, discriminant function analysis, multiple regression analysis, principal component analysis, selection indices, *Garcinia mangostana*.

Abstract: Studies on selection indices for activating seedling growth in mangosteen were conducted in the central orchard at the main campus of Kerala Agricultural University. The present investigation was undertaken with the main aim of identifying some of the basic reasons for slow growth in mangosteen, and to address this problem by developing and identifying criteria to select the age of the mother plant, fruit, seed and seedling characters or direct selection indices at all four stages with respect to seedling growth. Mother plants of four distinct age groups were used in the study. Variables were generated using all fruit, seed and seedling characters such as fruit index, seed index and seedling index by principal component analysis (PCA). Using PCA and multiple regression analysis, prediction models was fitted for the three indices. Major fruit, seed and seedling characters were identified by stepwise regression. Hierarchial analysis was performed based on Euclidean distance to find the similarities between the four age groups. Discriminant function analysis was performed and six discriminant functions were fitted with corresponding D^2 values to discriminate the six pairs involving the four age groups of the mother plants. For practical purposes, selection indices and best age group of mother plants are described in the work.

1. Introduction

Mangosteen (*Garcinia mangostana* L.), the queen of tropical fruits (Almeda and Martin, 1976; IBPGR, 1986; Kusumo and Verheij, 1994), is a very important crop of the warm humid tropics. The crop like other polyaxial tropical and sub tropical trees such as rubber, mango, cashew and citrus, exhibits a rhythmic growth habit under the relatively constant environmental conditions of the tropics (Alvim, 1964; Borchert, 1973). It bears profusely and fits very well as a component of home gardens of Kerala. Though it is a fruit crop with immense potential, both as a monocrop and as a mixed crop in coconut gardens with very high domestic and foreign demand, its cultivation on a commercial basis is limited by its long gestation phase (Wiebel *et al*., 1992 b).

The apomictic mangosteen (Richards, 1990; Normah *et al*., 1992) seedlings used for commercial planting are extremely slow growing, both at nursery stage and in orchards (Hume, 1947; Almeda and Martin, 1976; Wiebel *et al*., 1991, 1992 a). The slow growth rate and consequent long pre-bearing phase have been a cause for concern wherever mangosteen is grown. Though the long gestation period, ranging from 10-15 years (Lim, 1984; Richards, 1990; Wiebel *et al*., 1995), can be reduced by resorting to vegetative propagation, the problem of slow growth becomes all the more conspicuous (Wiebel *et al*., 1992 b, 1995). This crop lacks root hair (Richards, 1990) and this reduced vital link responsible for absorption of nutrients and water may be one of the prime reasons for slow growth. Low carbon acquisition capacity and prolonged dormancy of buds at the apex have also been listed as probable causes of the slow growth rate (Downton *et al*., 1990).

A critical review of the literature has revealed that only very few references to the crop exist. Selection index studies are available in palms but the aims differ as the thrust of the present work is directed toward the growth rate. This study was undertaken with the objectives of identifying some of the basic reasons for the slow growth rate and, secondly, to address this problem by identifying the mother plant, fruit and seed characters in relation to seedling growth and formulating the selection indices at these three basic levels. Accelerating the growth rate of mangosteen trees and thereby reducing the gestation period is one of the pre-requisites for an extensive commercialization of this crop.

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2. Materials and Methods

The materials and methodologies used in this investigation are presented separately.

Identification of mother plants belonging to various age groups

The first experiment on fixation of a selection index of Mangosteen (*Garcinia mangostana* L.) was based on the age of the mother plant and on fruit, seed and seedling characters. Hence a preliminary survey was conducted in the Kerala mangosteen growing tracts during the bearing phase ending with the onset of southwest monsoon. The main aim was to identify plants belonging to the different age groups as envisaged in the study.

The required number of plants belonging to different age groups could have been located in different regions, but the study was restricted to Pariyaram village of Chalakudi Taluk as age groups and numbers were available in a compact area. Hence the mother plants and fruit collection were centered on this area. Furthermore this approach aided in eliminating possible errors from an ecological point of view to the minimum.

Location of mother trees (Pariyaram village)

Pariyaram village is located at 10° 20' N latitude and 76° 26' E longitude, at an altitude of 3.25 m above mean sea level and 5 km (East) of the Chalakudi Railway Station, Thrissur District.

Climate and soil conditions of the area

The area receives an average rainfall of 2150 mm distributed over a period of a year. The mean maximum temperature ranges 28-36°C with a mean of 32°C. The mean minimum temperature ranges 12.8 to 20.6°C with a mean of 16.7°C. The relative humidity ranges from 90 to 98% with a mean of 94%.

The soil of the area where the mother trees are located is sandy alluvium with pH 5.5 -5.8 and belonging to the order of Entisol. The soil is low in available N and P_2O_5 and high in available K_2O.

Once fruit collection and observations were completed, the studies on seed germination and observations on seedling characters were carried out in the central orchard attached to the Department of Pomology and Floriculture, the College of Horticulture in the main campus of Kerala Agricultural University, Vellanikkara, Thrissur district, Kerala.

The experiment site experiences a warm humid tropical monsoon climate. It is situated at 12° 32'N latitude and at 74° 20'E longitude at an altitude of 22.5 m above mean sea level.

Soil type is a typical sandy clay loam with pH 5.4, EC 1.25 dsm^{-1}, and belonging to the order Ultisols.

Mother plant characters

Trees belonging to various age groups were identified and categorized into the four age groups as envisaged in this investigation:

1. Trees having an age of less than 25 years;
2. Trees having an age of 25-50 years;
3. Trees having an age of 51-75 years;
4. Trees having an age of more than 75 years.

Within each age group, ten fruits per tree were collected from five mother plants randomly, so that each age group had a total of fifty fruits. Care was taken to see that the mother plants under selection fell near the mid value of each age group and so that the five plants in a group were more or less uniform in growth characters (height and spread). Fruits were labeled individually and seeds were extracted from the fruits. The seeds were sown separately in black polythene bags (45x30 cm) filled with potting mixture made up of farmyard manure, sand and cow dung in the ratio of 2:2:1. The following observations on various fruit, seed and seedling characters of the four age groups (Table 1) were recorded separately as presented below.

Fruit characters

Fruit weight, fruit girth, fruit volume, pulp weight, rind weight, number of segments, number of seedless fruits, number of one-seeded fruits, number of two-seeded fruits, number of three- or more seeded fruits, number of seedlings produced per fruit and seed specific gravity were recorded.

Seed characters

Observations on seed weight, seed length, seed thickness at centre, seed volume, seed specific gravity, number of seeds per fruit, number of seedlings per seed, number of ungerminated seeds, number of seeds producing one seedling, number of seeds producing two or more than two seedlings (Table 2), number of days taken to germination, and germination rate were recorded based on ISTA guidelines (ISTA, 2003).

Seedling characters

Height, girth at collar, total number of leaves, and survival rate of the seedlings at three months, six months, nine months and one-year stage after germination were recorded. Increment in height, girth, and total number of leaves at quarterly intervals up to one year were computed. Number of new flushes per year, number of leaves/flush, and total leaf area were limited to a twelve-month stage.

Shoot and root characters

Shoot and root weight, dry weight, root to shoot dry weight ratio, length of longest root, number of primary, secondary and tertiary roots, and total number of roots were recorded. Based on the fruit index, seed index and seedling index the selection indices were determined.

Biochemical analysis

The following biochemical characters were estimated in seeds and leaves of the mother plants and seedlings of the four age groups. However biochemical analyses were restricted to the treatments showing the growth of best, in-

Table 1 - Latent vectors and variance of the principal component analysis performed for generating fruit, seed and seedling indices separately in mangosteen (*Garcinia mangostana* L.)

Sl. No.	Characters	Principle component 1	Principle component 2
	Fruit characters		
1	Fruit weight	0.62	0. 14
2	Fruit volume	0.62	0 .14
3	Fruit girth	0.02	0.00
4	No. of segments	0.00	0.00
5	Pulp weight	0.15	0.16
6	Rind weight	0.47	-0.62
7	Fruit specific gravity	0.00	0.00
8	Number of seedless fruits	0.00	0.00
9	Number of one seeded fruits	0.00	-0.01
10	Number of two seeded fruits	0.00	0.0 1
11	Number of three and multi seeded fruits	0.00	0.01
12	Number of seedlings/Fruit	0.00	0.04
	Cumulative variance	**96.27**	**98.68**
	Seed characters		
1	Seed weight	0.00	-0.07
2	Seed length	0.00	-0.04
3	Seed thickness at center	0.00	-0.03
4	Volume	0.00	-0.08
5	Seed specific gravity	-0.01	0.1 I
6	Number of seeds	0.00	-0.04
7	Number of seedling/seed	0.00	0.00
8	Number of days to germination	0.02	0.99
9	Seed with one seedling	0.00	0.00
10	Seed with twin and multiple seedlings	0.00	0.00
11	Germination percentage	1 .00	-0.01
	Cumulative variance	**97.63**	**99.50**
	Seedling characters		
1	Height	0.03	-0.01
2	Girth at collar	0.00	0.00
3	Total number of leaves	0.03	0.00
4	Number of flushes/year	0.00	0.00
5	Total leaf area	0.98	-0.21
6	Survival rate at twelth month (%)	0.04	0.18
7	Shoot fresh weight	0.01	0.06
8	Root fresh weight	0.01	0.03
9	Total fresh weight	0.02	0.09
10	Shoot dry weight	0.01	0.03
11	Root dry weight	0.00	0.01
12	Total dry weight	0.01	0.04
13	Root to shoot dry weight ratio	0.00	0.00
14	Root length (longest root)	0.03	0.1 8
15	Total number of roots	0.20	0.94
	Cumulative variance	**70.05**	**95.77**

Table 2 - Average fruit index, seed index and seedling index values of four age groups in mangosteen (*Garcinia mangostana* L.)

Age groups (Years)	Fruit index	Seed index	Seedling index
<25	90.55	80.54	93.69
25-50	88.62	88.36	101.17
51-75	132.90	84.47	65.20
>75	110.42	62.12	67.17
Average	102.16	81.58	86.21

termediate and least categories, in the experiments of activation of seedling growth using growth regulators and *A M* fungi separately.

1. Nitrogen - seeds and leaves
2. Phosphorus - seeds and leaves
3. Potassium - seeds and leaves
4. Protein - seeds and leaves
5. Sodium - seeds and leaves
6. Chlorophyll - leaves only
7. Total sugar content - seeds only
8. Total carbohydrates - seeds and leaves
9. Total phenols - seeds and leaves
10. Abscisic acid - seeds and leaves

Selection index

For this experiment, fully matured (dark purple colour) fruits were harvested randomly and the following observations were taken.

Fruit characters

Individual fruits were labeled immediately after harvest. Fruit weight was then recorded using an electronic balance (Contech precision balance) and the average expressed in grams. Girth of the fruit was measured using a thread and its length measured using a metre scale and the average expressed in centimeters. Fruit volume was measured by water displacement method and the average volume expressed in milliliters. The fruit hull was carefully removed and the weight of the white-segmented pulp (aril) of each fruit with seeds was measured using an electronic balance. Seeds were extracted and their weight recorded and the average expressed in grams. Pulp weight of each individual fruit alone was calculated using the formula:

Pulp weight alone (g) = Pulp weight with seed (g) - seed weight alone (g)

The rind weight of each individual fruit was calculated using the following formula and the average expressed in grams:

Rind weight (g) = Total fruit weight (g) - Pulp weight with seed (g)

Numbers of white juicy segments were counted immediately after the fruits were opened and the average

expressed as a number. Numbers of seeds per fruit were counted and categorized as one, two, three and more in three-seeded and seedless fruits and the average expressed as a number.

Total number of seedlings obtained from individual fruits was counted irrespective of the number of seeds per fruit and the average was expressed numerically.

The specific gravity fruits was calculated using the following formula and the average expressed as grams/milliliter:

Fruit specific gravity = (Fruit weight / Fruit volume)

Seed character

The seeds were extracted from the pulp and the weight of individual seeds in fruits was measured using an electronic balance; the average seed weight was expressed in grams. Seed length and thickness at the centre were also measured, with the aid of a meter scale, and averages expressed in centimeters. Seed volume was measured by water displacement method and the average expressed in milliliters. Seed specific gravity was calculated using the formula

Seed specific gravity = (seed weight / Seed volume)

and the average expressed as grams/milliliter.

Total number of seeds present in individual fruits, total number of seedlings produced by individual seeds and total number of ungerminated seeds were counted and averages expressed as a number. The number of seedlings produced by an individual age group was calculated and the average expressed as a number. The number of seedlings produced by individual seeds was counted and categorized as seeds producing one, two and more than two seedlings and the average expressed as a number.

The number of days from date of sowing to seed germination was counted and the average expressed as number of days. Germination rate was calculated for each individual age group and the average expressed as a percentage.

Germination % = (Total number of seeds germinated / Total number of seeds sown) x 100

Seedling characters

Seedling height was measured from the collar region to the tip of the main stem using a meter scale and expressed in centimeters.

Seedling girth was measured at the collar using a thread; thread length was measured using a meter scale and averages expressed in centimeters. Total number of leaves produced by an individual seedling was counted and the average expressed as a number.

The increments were calculated by computing the difference between two consecutive values of the particular interval and the average expressed in centimeters.

The number of flushes and number of leaves/flush produced by an individual seedling were counted and the average expressed as a number.

Leaf area was calculated by multiplying the length, the breadth and the factor (0.6727) and the average expressed as cm^2. The factor was pre-standardized, for this purpose, by measuring the length and breadth of 100 leaves: the leaf area of the corresponding leaf was measured by leaf area meter to work out the factor value. Thus, the factor value (0.6727) was derived using the formula:

Factor = (Leaf area / Length x breadth)

Survival rate was determined from the number of established plants as a percentage of the total number of seedlings observed after germination.

Survival % = (Total number of seedings observed at each interval / Total number of seedlings observed immediately after germination) x 100

Shoot and root characters

Seedlings were uprooted one year after germination. The plants were immediately cut and separated into shoots and roots. Fresh shoot and root weights were recorded separately using an electronic balance and the average expressed in grams.

The samples collected for the fresh weight were dried in an oven at 60°C till the weight of the samples remained constant. Dry weights were recorded separately and averages expressed in grams.

Dry weight ratio of root to shoot was calculated by the formula:

Dry weight ratio = (Root dry weight / Shoot dry weight)

Length of the longest root (taproot) was measured from the collar region to the growing tip of the taproot using a meter scale and expressed in centimeters. After carefully removing the potting mixture using water spray, the number of primary, secondary, tertiary roots, and total number of roots were counted and their averages expressed as the number of roots.

Biochemical studies

Leaf samples from seedlings were collected one year after germination; leaf samples from mother plants were also collected. The third leaf from the tip was collected and oven dried at 60°C, ground and used to estimate the content of N, P, K and Na.

The total nitrogen content of leaf samples was determined by microkjeldhal method (Jackson, 1973) and the average expressed as percentage. The phosphorus content of the samples were determined using the di-acid extract method (Jackson, 1973). A spectrophotometer was used to determine colour intensity developed by Vanado-molybdo phosphoric yellow colour method and readings were taken at 420nm wavelength. Phosphorous content was cal-

culated using a standard graph and the average expressed as a percentage. The potassium and sodium contents of samples were determined with di-acid extract (Jackson, 1973) and read in an EEL flame photometer, at 548 nm and 598 nm respectively, and averages expressed as a percentage. Nitrogen content was estimated by microkjeldhal method (Jackson, 1973) and the value of nitrogen content was multiplied with the factor 6.25 to get the crude protein content and the average expressed as a percentage.

Chlorophyll content (total chlorophyll, chlorophyll a and chlorophyll b) was estimated in leaf samples by Arnon's Acetone method (Sadasivam and Manickam, 1996) and the average expressed in milligrams.

Total sugars were estimated using standard procedure (A.O.A.C., 1980), total carbohydrates were estimated using Anthrone method (Dubois *et al*., 1951), and averages expressed in milligrams; total phenol content was estimated using the Folin-Ciocalteau method (Sadasivam and Manickam, 1996) and expressed in milligrams.

Procedure adopted for quantification of abscisic acid

The procedure adopted for quantification of abscisic acid was a modification of the standard method of Little *et al*. (1972). The modification became imperative as bands were not obtained. The procedure was standardized and bands were obtained corresponding to the standard abscisic acid. Further quantification was done using a U-V spectrophotometer and standards of known concentration from which a standard graph was obtained.

Generation of new varieties as index

Variability in morphology characters (38 characters) were recorded. Principal component analysis (PCA) was carried out independently for 12 fruit, 11 seed and 15 seedling characters in order to establish a list of minimum descriptors (A.O.A.C., 1980) making it possible to identify the best age group of mother plants. The first principal component which accounted for maximum possible variance was selected. This is supported by the work of Manzano *et al*. (2001).

Estimation of correlation

Associations between the various characters were made using the Karl Pearsons product movement correlation coefficient (r). Correlations between age of mother plant, fruit characters, seed characters, seedling characters, fruit index, seed index and seedling index were calculated according to the method of Searle (1961).

Principal component analysis

The observations of 12 fruit, 11 seed and 18 seedling characters of the four age groups and their corresponding index values (i.e. fruit, seed and seedling) of the 135 plants were used for the study. The volume of the data had to be reduced first for the sake of simplicity. This was based on the fact that principal component analysis is one of the variable-directed techniques aimed at reducing dimensionality of the problem and which finds new variables that make the data easier to understand (Chatfield and Collins, 1980).

Principal component analysis (PCA) was used to determine the relationship among the fruit characters, seed characters, seedling characters and four age groups (Meilgard *et al*., 1991) and was performed in XLSTAT version 5.1v2 package. It was also used to provide a graphical description of the characteristics. Factor scores were calculated for each fruit, seed and seedling characteristics using the formula:

Factor scores= each attribute factor loading x the original attribute mean score

Although the characteristic factor scores were calculated from a single analysis, they were plotted on separate figures to facilitate interpretation. These figures then provided a visual representation of the dominant age groups and characters for each fruit, seed and seedling character (Dever *et al*., 1996).

Step-wise regression

To facilitate the prediction of indices reduction in number of variables is imperative. Hence the step-wise regression procedure was adopted to identify major characters, which can be used to predict the fruit, seed and seedling indices. Furthermore, adoption of step-wise regression has been scientifically established to reduce multi-collinearity among the independent variables and to arrive at the best subset of variables (Draper and Smith, 1966).

Cluster analysis

Cluster analysis was performed to find the similarities (or dissimilarities) between the four age groups based on absolute square Euclidean distance (Johnson and Wichern, 1998). The clusters and square Euclidean distances (Chatfield and Collins, 1980) were graphically represented by dendrograms (Manzano *et al*., 2001). The Euclidean distance was calculated using the formula

$$Drs : \{\sum^{P} (X_{ri} - X_{si})^2\} \text{ to } ½$$

- and the variable were the Principal component co-ordinates.

where Drs - Distance from individual r to individual s, X_{rj} - Value of individual r X_{sj} - Value of individuals.

Selection index

Selection index or total index values were worked out using principal component analysis. Principal component analysis was performed on all the fruit, seed and seedling characters collectively and the first principal component was taken as the index value for selection, as in the method described above. Regression analysis was performed for each age group separately to find the best age group with equal weightage of all fruit, seed and seedling characters. Then the best age group or selection index (total index)

was predicted using the regression equation with the fruit, seed and seedling indices as the explanatory variables. The predictor equation was found to be

$$Y=a_1F1 + a_2S1 + a_3SDLI + C$$

where a_1, a_2, a_3 were regression coefficient or weightage of F1, S1 and SDLI, which are fruit index, seed index and seedling C the Intercept constant (Manoj, 1992).

3. Results

The results of the study have been divided into broad aspects for presentation.

Fruit, seed and seedling indices

The numbers of independent variables were large in the present study. Hence, to reduce the number of descriptors, principal component analysis was performed using the deviation squares and products matrix of the 12 fruit, 11 seed, and 15 seedling characters independently in order to establish a list of minimum descriptors (i.e. fruit index, seed index and seedling index), thereby enabling identification of the best age group of mother plants, as well as reducing the complexity of the statistical analysis. The first principal components of fruit, seed and seedling characters, which accounted for the cumulative variance of 96.27, 97.63 and 70.05% respectively, are given in Table 1. The computed average fruit, seed and seedling index values of each age group are presented in Table 2 and figure 1.

Fruit index

The highest fruit index value was in the age group of 51-75 years of mother plants, followed by the age group of more than 75 Years.

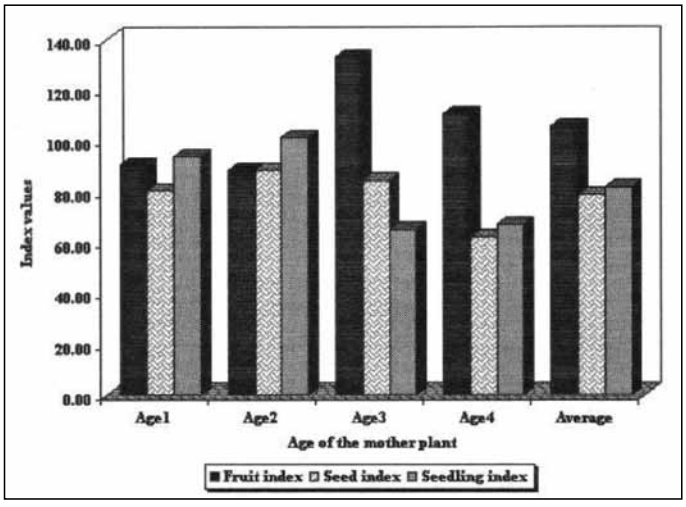

Fig. 1 - Fruit, seed and seedling index of four age groups in mangosteen (*Garcinia mangostana* L.).

Seed index

The highest seed index value was recorded in the 25-50 year age group, followed by 51-75 years. The lowest seed index values were observed in the more than 75 years age group.

Seedling index

The highest seedling index was observed in the age group 25-50 years, followed by the age group of less than 25 years. An important observation was made: the difference between the best group (25-50 years) and the two age groups of more than 50 years was near to one and a half times more.

Index values observed for fruit, seed and seedling characters differed but the trend was the same for the seed and seedling characters. However, equal importance was given to each of the three index values and to the age group for finally determining the best age group of mother plant.

Principal component analysis

Principal component analysis was performed using the deviation squared and product matrix of the 14 fruit characters, 13 seed characters, 20 seedling characters, the age of the mother plants and all the three fruit, seed and seedling indices separately. The first two principal components, which accounted for the cumulative variance of fruit, seed and seedling characters, were found to be 88.78, 92.94 and 92.49% respectively. The cumulative variance, factor scores and contribution of variation of each character are presented in Table 3 and the same for each age group in Table 4. The fruit index among the fruit characters, seed index among the seed characters and seedling index among the seedling characters were predicted using the regression equation with the first two principle components as the explanatory variables. The predictor equations were found to be

$$Y = 0.0695\ P_1 - 0.1283\ P_2 - 0.0197 \text{ with an } R^2 \text{ of } 0.999$$

Where Y_1 P_1 and P_2 are the fruit index first and second principal components respectively;

$$Y = 0.3198\ P_1 + 0.6307\ P_2 - 0.0135 \text{ with an } R^2 \text{ of } 1.00$$

Where Y_1 P_1 and P_2 are the seed index, first and second principal components respectively;

$$Y = 0.7003\ P_1 - 0.0703\ P_2 + 3.263 \text{ with an } R^2 \text{ of } 0.999$$

Where Y_1, P_1 and P_2 are the seedling index first and second principal components respectively.

Also the relationship and dominance of the fruit, seed and seedling characters including four age groups of the mother plants and three (fruit, seed and seedling) indices separately were determined using factor scores. The depiction of the various characters of factor I showed negative scores for characters such as number of seeds per fruit, specific gravity and selection index, whereas factor II revealed positive scores only for seedlessness and seed specific gravity (Table 3). Distinct variations in the dominant

characters were observed between the various age groups. In the case of fruit characters, fruit index, fruit weight, fruit girth, fruit volume, pulp weight, number of segments and rind weight characters of age group three (51-75 years) were dominant. On the other hand, the number of seeds per fruit was observed as the dominant characters in the

Table 3 - Eigen vectors, factors loadings and contribution of variations by each character in fruit, seed and seedling characters in mangosteen (*Garcinia mangostana* L.)

Characters	Eigenvectors		Factor loadings		Contributions of variation (%)	
	F1	F2	F1	F2	F1	F2
Fruit characters						
Fruit weight	0.30	-0. 17	0.96	-0.28	9.27	3.01
Girth	0.21	-0.07	0.66	-0.11	4.44	0.44
Volume	0.29	-0.24	0.92	-0.39	8.50	5.84
Pulp weight	0.21	-0.46	0.66	-0.73	4.39	20.90
Rind weight	0.31	-0.05	0.99	-0.08	9.86	0.25
Segments	0.30	-0.14	0.95	-4.22	9.14	1.94
Seeds	-0.28	-0.20	-0.87	-0.32	7.70	3.97
Seedless fruits	0.29	0.27	0.90	0.43	8.20	7.08
One seeded fruits	-0.27	-0.24	-0.84	-0.39	7.22	5.98
Two seeded fruits	-0.21	-0.18	-0.65	-0.29	4,29	3.37
Three and >three seeded fruits	-0.27	-0.31	-0.84	-0.49	7.16	9.32
Specific gravity	-0. 11	0.58	-0.34	0.94	1.16	34.01
Fruit index	0.31	-0.06	0.97	-0.10	9.54	0.41
Selection index	-0.30	-0.19	-0.95	-0.30	9.13	3.49
Cumulative variance	**70.37**	**88.79**				
Seed characters						
Seed weight	0.29	-0.28	0.82	-0.55	8.29	7.81
Seed length	0.34	-0.02	0.98	-0.04	11.68	0.03
Thickness at center	0.31	0.06	0.89	0.11	9.68	0.31
Seed volume	0.29	-0.27	0.84	-0.54	8.69	7.40
Specific gravity	-0.28	0.29	-0.80	0.57	7.81	8.17
Number of seedlings / seed	0.32	0.07	0.90	0.14	9.94	0.49
Ungerminated seeds	-0.30	-0.22	-0.87	-0.44	9.30	5.04
Seeds producing one seedlings	0.26	0.27	0.74	0.53	6.69	7.23
Seeds producing 2 and >2 seedlings	0.33	0.10	0.95	0.19	11.05	0.94
Days to germination	-0.24	0.31	-0.68	0.62	5.72	9.81
Germination %	0.24	0.37	0.68	0.73	5.59	13.73
Seed index	0.20	0.41	0.57	0.82	4.01	17.04
Selection index	-0.12	0.47	-0.35	0.93	1.54	21.99
Cumulative variance	**62.79**	**92.94**				
Seedling characters						
Height	0.26	0.09	0.99	0.15	6.85	0.88
Girth	0.26	-0.09	0.97	-0.13	6.62	0.74
Total leaves	0.26	0.08	0.97	0.12	6.60	0.59
New flushes / year	0.10	0,55	0.39	0.85	1.09	29.76
Total leaf area	0.25	0.19	0.95	0.30	6.35	3.63
Survival rate	0.2l	-0.36	0.78	-0.55	4.27	12.70
Shoot fresh weight	0.23	0.06	0.87	0.09	5.34	0.33
Root fresh weight	0.26	0.05	0.99	0.08	6.87	0.23
Shoot dry weight	0.24	-0.04	0.90	-0.06	5.64	0. 14
Root dry weight	0.26	0.03	0.99	0.04	6.89	0.08
Root to shoot dry weight ratio	-0.14	0.47	-0.54	0.72	2.08	21.65
Root length	0.23	-0.16	0.86	-0.25	5.23	2.68
Primary roots	0.25	-0.15	0.94	-0.23	6.18	2.29
Secondary roots	0.26	-0.01	1.00	-0.02	7.01	0.02
Tertiary roots	0.14	0.49	0.55	0.76	2.09	24.22
Total roots	0.26	0.01	1.00	0.02	7.01	0.02
Seedling index	0.26	-0.01	0.99	-0.02	6.93	0.02
Selection index	0.26	-0.01	0.99	-0.02	6.93	0.02
Cumulative variance	**79.13**	**92.49**				

less two age groups (i.e. <25 and 25-50 years age groups). Interestingly, in age group four (>75 years), the most dominant character was observed to be seedlessness (Fig. 2).

Table 4 - Factor scores and contribution of variations by each age group based on the fruit, seed and seedling characters in mangosteen (*Garcinia mangostana* L.)

Characters	Factor scores		Contributions of age groups {%}	
	F1	F2	F1	F2
Fruit characters				
Age 1	-1.74	-0.62	6.14	2.99
Age 2	-4.34	-0.66	38.27	3.39
Age 3	4.96	-1 .76	49.91	24.02
Age 4	1.60	3.00	5.21	69.59
Mean	-0.48	0.05	0.47	0.02
Seed characters				
Age 1	-1.64	1.04	6.59	5.53
Age 2	-0.64	2.87	0.99	41 .98
Age 3	5.26	-1.13	67.89	6.55
Age 4	-3.16	-2.99	24.46	45.70
Mean	0.17	0.22	0.07	0.24
Seedling characters				
Age 1	3.35	0.92	15.73	7.10
Age 2	4.93	-0.91	34.14	6.95
Age 3	-4.52	-2.27	28.75	42.95
Age 4	-3.90	2.27	21 .35	43.00
Mean	0. l5	-0.01	0.03	0.00

The depiction of the various seed characters of factor I showed negative scores for seed specific gravity, ungerminated seeds, days to germination and selection index. In the case of factor II the negative scores were found for seed weight, seed length, seed volume and ungerminated seeds. All other characters showed positive factor scores. In the case of seed characters, also variations in dominant characters were observed with respect to age groups. The seed index, selection index, germination percentage, seeds producing one seedling and number of seedlings per seed were the dominant characters observed in the second age group (25-50 years). In age group one (<25 years) the number of days taken to germination and seed specific gravity were noted to be the dominant characters. In age group three, seed weight, seed volume and seed length were the dominant characters, whereas in age group four, the dominant character was the number of ungerminated seeds (Fig. 3).

In the case of seedling characters, negative scores were observed only for the root to shoot dry weight ratio in factor I; in factor II negative scores were found for girth, survival rate, shoot dry weight, root length and number of roots (primary, secondary and tertiary), seedling index and selection index. All other characters showed positive factor scores. A study of the dominant characters of seedlings revealed that the major characters (seedling index seedling girth, total number of roots, number of primary, secondary roots, survival rate, shoot, fresh weight, dry weight, root dry weight, height of the seedlings and selection index were the dominant characters in age group two. In the case of age group one characters such as the root fresh

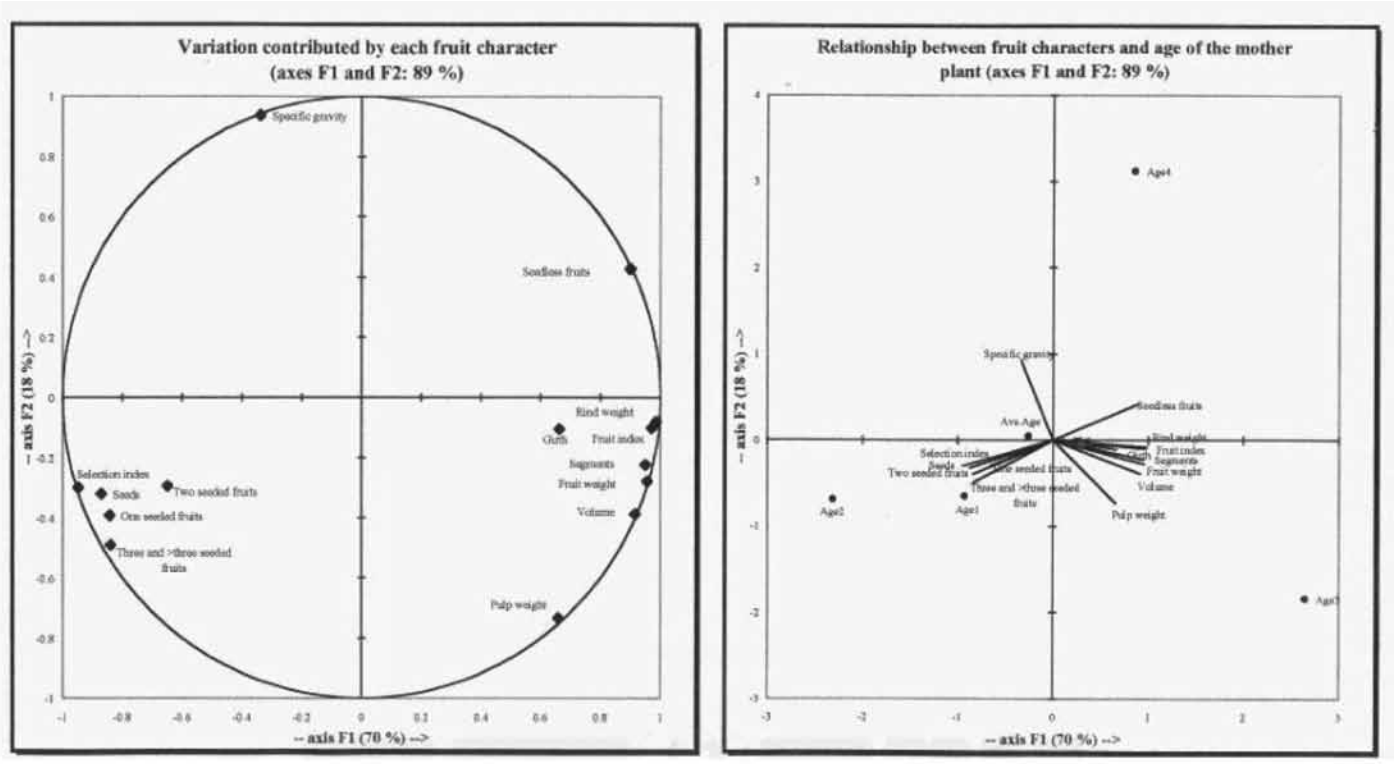

Fig. 2 - Plot showing the percentage of variation contributed by each fruit character and the relationship between age of the mother plant and fruit characters in mangosteen (*Garcinia mangostana* L.).

weight, total number of leaves, number of tertiary roots and new flushes per shoot were the dominant characters. In the higher two age groups, no specific dominance were observed (Fig. 4).

Principal component analysis reduces only the dimensionality, but not the number of variables involved. Hence, to reduce the number of variables and to identify the major

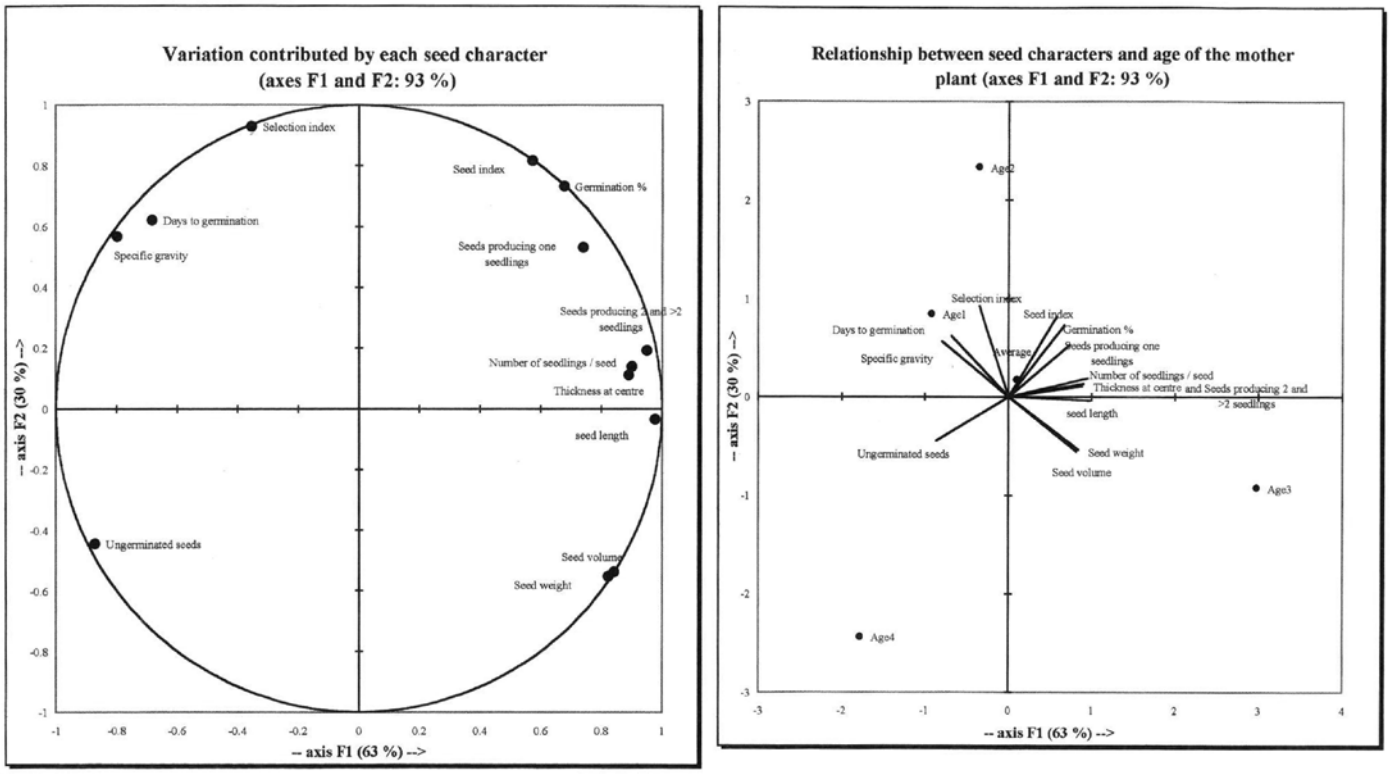

Fig. 3 - Plot showing the percentage of variation contributed by each seed character and the relationship between age of the mother plant and seed characters in mangosteen (*Garcinia mangostana* L.).

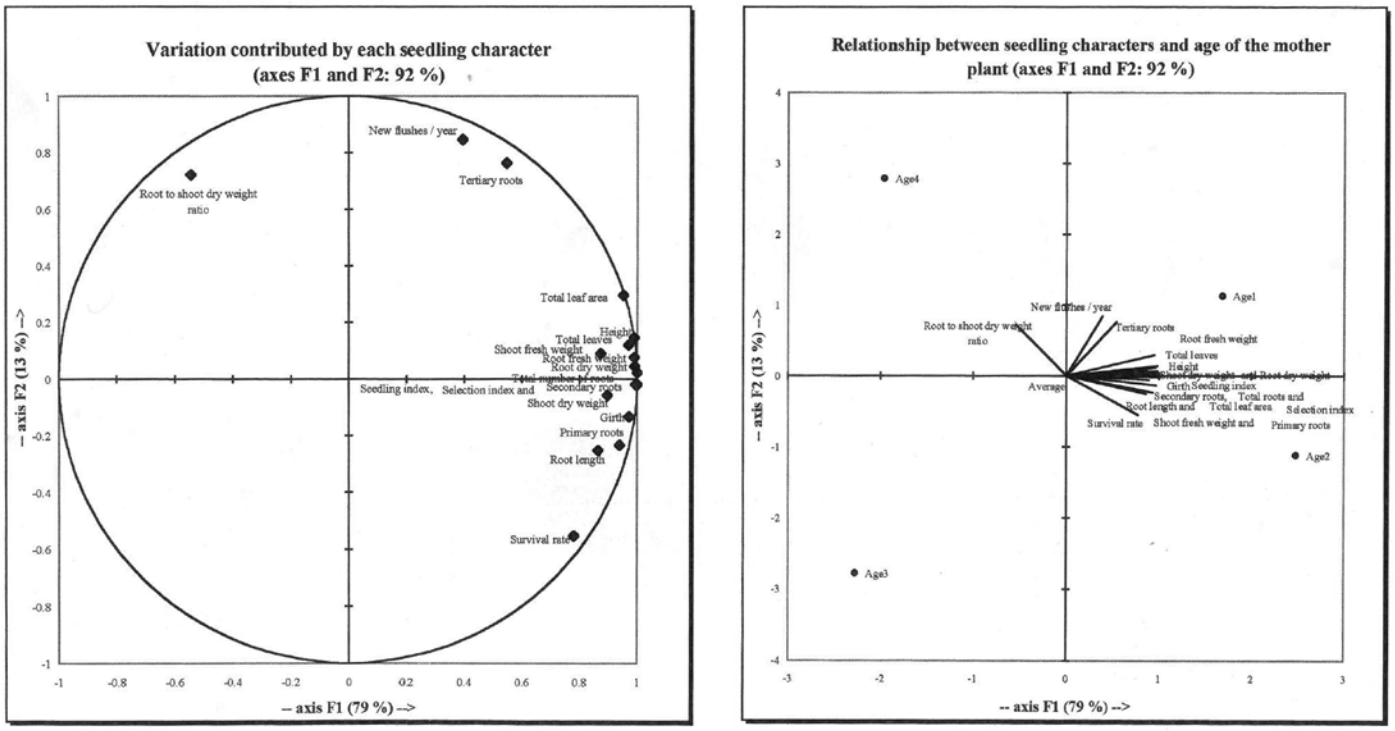

Fig. 4 - Plot showing the percentage of variation contributed by each seedling character and the relationship between age of the mother plant and seedling characters in mangosteen (*Garcinia mangostana* L.).

variables that contribute greater variation to the fruit, seed and seedling index, a step-wise regression was performed.

Step-wise regression

Step-down regression of the fruit index on 13 fruit characters, seed index on the same 13 fruit characters, seed index on the 12 seed characters separately and all the 25 fruit and seed characters collectively, and seedling index on 13 fruit characters, 12 seed characters, 19 seed characters including age separately and also collectively (all the 42 fruit, seed, seedling characters and age) was carried out and the following regression equations with major variable, to each, indices on each group of characters, were observed.

Fruit index on fruit characters alone

$Y = 1.295\ x_7 + 1.7\ l5x_8 + 1.3\ 19\ x_{14} - 4.17$ with an R^2 at 0.993

where Y - fruit index, x_7 - pulp weight, x_8 - rind weight, x_{14} - number of seedlings per fruit.

Seed index on fruit characters alone

Y = -a.282 x2 + 0.427 x3 + 14.416 x5 + 22.368 x10+ 126.73 with an R2 at 0.203.

Where Y – seed index, x2 – age of the mother plant, x_3 - fruit weight, x_5 - fruit girth, x_{10} - number of seedless fruits.

Seed index on seed characters alone

Y= 0.007 x15 - 0.004 x16 - 0.012 x17 + 0.009 x18- 0-008 x19 + 0.002 x20 +0.004 x21 + 0.015 x22 + 0.002 x24 + x25 + 0'01 with an R2 at 1.00

where y - seed index, x_{15}- seed weight, x_{16} - seed length, x_{17} - seed thickness at centre, x_{18} - seed volume, x_{19} - seed specific gravity, x_{20} - number of seeds per fruit, x_{21} - number of seedling per seed, x_{22} - number of days to germination, x_{24} - number of seeds producing two or more than two seedlings, x_{25} – germination percentage.

Seed index on fruit and seed index collectively

$Y = -0.002\ x_{11} - 0.006\ x_{15} - 0.006\ x_{17} + 0.006\ x_{18} - 0.007\ x_{19} + 0.005\ x_{21} + 0.015\ x_{22} - 0.002\ x_{23} + x_{25+}\ 0.01$ with an R^2 at 1.00

where y - seed index, x_{11} – number of one seeded fruits, x_{15} - seed weight, x_{17}- seed thickness at centre x_{13} - seed volume, x_{19} - seed specific gravity, x_{21} - number of seedling per seed, x_{22} - number of days to germination, x_{23} - seed with one seedling x_{25} - germination percentage.

Seedling index on fruit characters including age

Y =-0.481 x2 + 107.15 with an R2 of 0.0388

Where, Y - seedling index, x_2 - age of the mother plant.

Seedling index on seed characters

Y =136.67 x17 - 1083 with an R2 of 0.1087

Where, Y - seedling index, x_{17} - seed thickness at centre.

Seedling index on seedling characters

Y= 0.979 x30 + 0.037 x31 + 0.042 x32+ 0.027 x33 + 0.031 x39+ 0.202 x40 + 0 .36 with an R2 of 1.00

where, Y - seedling index, x_{30} - total leaf area per seedling, x_{3l} - survival rate at l2-months stage, x_{32} – shoot fresh weight, x_{33} - root fresh weight, x_{39} - foot length, x_{40} - total number of roots.

Seedling index on fruit, seed and seedling characters collectively including age of mother plant

Y= 0.026 x26 + 0.030 x28+ 0.977 x30 + 0.037 x31 + 0.0034 x32- 0.029 x33+ 0.017 x35 + 0.009 x36 + 0.031 x_{39} + 0.031 x_{39} + 0.201 x_{40} with an R^2 of 1.00

where, Y - seedling index, x_{26} - height of the seedling, x_{28} - total number of leaves, x_{30} - total leaf area, x_{31} - survival rate at twelve-months stage, x_{32} - shoot fresh weight, x_{33} - root fresh weight, x_{35} - shoot dry weight, - root dry weight, x_{36} - root dry weight, x_{39} - root length, x_{40} - total number of roots.

Cluster analysis

Cluster analysis was performed to highlight the similarities and differences based on the fruit, seed and seedling characters separately and all characters collectively among the four age groups. Absolute Euclidean distances between the four age groups were calculated based on the fruit, seed and seedling characters and are presented in Table 5. Figure 5 illustrates the dendrogram showing the clusters (or similarities) based on the fruit, seed and seedling characters among the four age groups.

Considering fruit characters, closest distance was observed between age groups of more than 25 years and 25-50 years, followed by the age group of 25-50 years and more than 75 years; in all the remaining combinations greater distances were observed, indicating that the age group of more than 25 years and 25-50 years have more similar fruit characters.

With regard to seed characters, closest distance was again observed between age groups of more than 25 years and 25-50 years, followed instead by the age group of more than 25 years and more than 75 years. With regard to all the remaining combinations greater distances were observed, indicating that the age group of more than 25 years and 25-50 years have more similar seed characters.

For seedling characters, closest distance followed the same trend (i.e. age group of more than 25 years and 25-50 years). All the remaining combinations were observed at greater distances, indicating that also for seedling charac-

Table 5 - Proximity matrix showing the absolute squared Euclidean distance between four age groups based on fruit, seed, seedling characters separately and collectively in mangosteen (*Garcinia mangostana* L.)

Characters and age groups	Absolute squared Euclidean distance			
	1	2	3	4
Fruit				
1	0.00	14.41	39.25	20.57
2	14.41	0.00	3 1.60	15.6
3	39.25	31.60	0.00	22.57
4	24.57	15.60	22.57	0.00
Seed				
1	0.00	13.14	30.09	13.24
2	13.14	0.00	21.58	20.37
3	30.09	21.58	0.00	33.59
4	13.24	20.37	33.59	0.00
Seedling				
1	0.00	5.65	29.97	35.44
2	5.65	0.00	26.48	34.63
3	29.97	26.48	0.00	11.83
4	35.44	34.63	11.83	0.00
All characters				
1	0.00	39.06	109.58	77.77
2	39.06	0.00	100.31	90.23
3	109.58	100.31	0.00	75.05
4	77.77	90.23	75.05	0.00

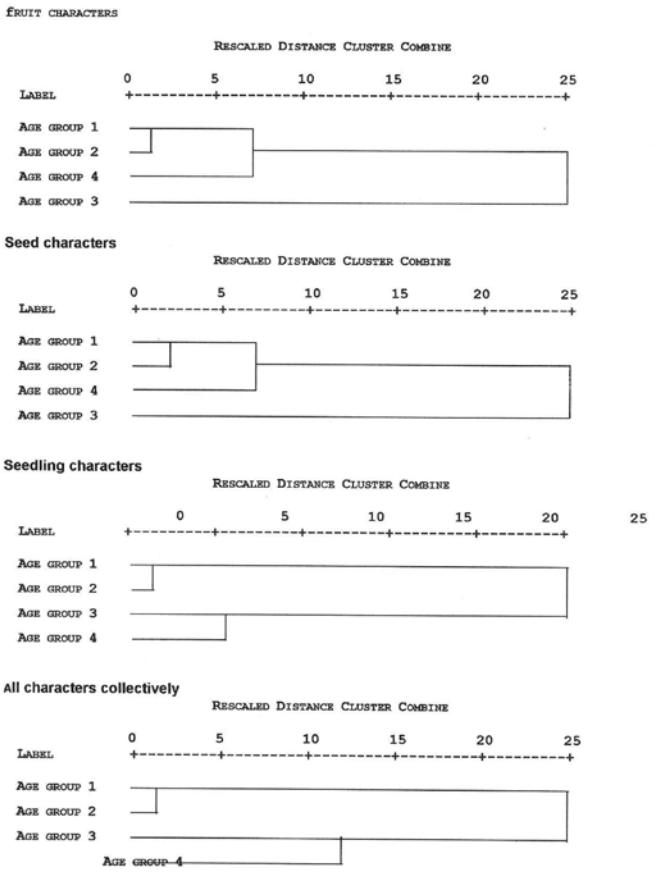

Fig. 5 - Dendrogram showing the similarity and proximity of the four age groups based on the fruit, seed and seedling characters individually and collectively in mangosteen (*Garcinia mangostana* L.).

ters the age group of more than 25 years and 25-50 years are more similar.

When the fruit, seed and seedling characters were taken collectively, the closest distance was observed between age group of more than 25 years and 25-50 years, followed by the age group of more than 25 years and more than 75 years; in all the remaining combinations greater distances were observed. Finally it may be confirmed that, based on all four dendrograms, maximum similarity for all the characters was between the age group of less than 25 years and 25-50 years.

Discriminant function analysis

Fruit index, seed index and seedling index of the four age groups were discriminated using discriminant functional analysis. Discriminant functions were fitted for discrimination pairs of these age groups. The functions derived were as follows.

For groups 1 and 2
$Z = 0.0004\ FI - 0.0547\ SI - 0.0004\ SDLI$ with $D^2 = 0.4313$

For groups 1 and 3
$Z = -0.0483\ FI - 0.0313\ SI + 0.0075\ SDLI$ with $D^2 = 2.381$

For groups 1 and 4
$Z = -0.0183\ FI - 0.0680\ SI + 0.0047\ SDLI$ with $D^2 = 1.739$

For groups 2 and 3
$Z = -0.0406\ FI + 0.1264\ SI + 0.0137\ SDLI$ with $D^2 = 2.782$

For groups 2 and 4
$Z = -0.0265\ FI + 0.3508\ SI + 0.0150\ SDLI$ with $D^2 = 10.29$

For groups 3 and 4
$Z = 0.0024\ FI + 0.2152\ SI + 0.0004\ SDLI$ with $D^2 = 4.064$

where FI - the fruit index, SI - the seed index and SDLI - the seedling index are the explanatory variables.

The average values of fruit index, seed index and seedling index for each age group were fitted in the equation and the discriminant values calculated for each pair of age groups. The direction of association of the discriminant coefficients in each age group is given in Table 6. In this maximum number of positive directions were observed in the age group of 25-50 years, when discriminated with other three

Table 6 - Direction of association of six discriminant function coefficients involving the four age groups in mangosteen (*Garcinia mangostana* L.)

Pairs of compared age groups	Magnitude of the values		
	Fruit index	Seed index	Seedling index
1 and 2	+	-	-
1 and 3	-	-	+
1 and 4	-	+	+
2 and 3	-	+	+
2 and 4	-	+	+
3 and 4	+	+	-

age groups (Table 7). The corresponding discriminant values of each age group (criteria) and the mid values of each pair of discriminant values were taken to draw the conclusion that, if the values is below the mid values it falls in the group I, if not it falls in the Group II.

The criterion and criteria are presented in Table 8.

Selection index

Principal component analysis was performed on all the fruit, seed and seedling characters collectively and first principal component was taken as the index value for selection (Fig. 6). The selection index was predicted using the multiple regression equation constant for each age group independently and also the average of all age groups. The predictor equation was found to be

For the less than 25 years group

$Y = -0.0402\ FI + 0.0513\ SI + 0.9960\ SDLI - 0.4317$ (adj. $R^2 = 0.99$)

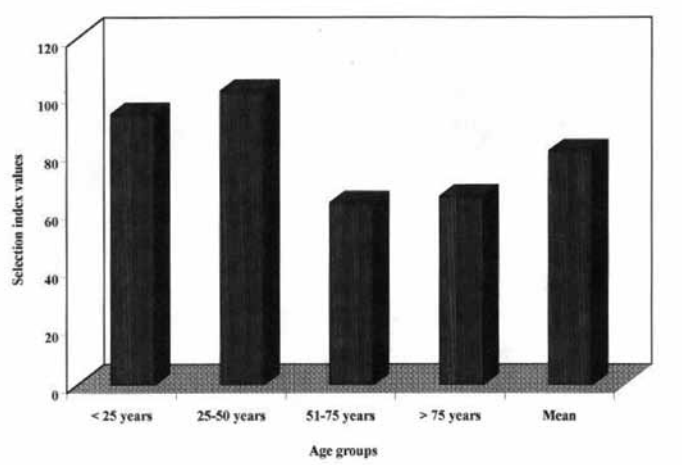

Fig. 6 - Selection index or total index values of all the four age groups in mangosteen (*Garcinia mangostana* L.).

Table 7 - Direction of association of six discriminant function coefficients when used to compare each age group with the remaining three age groups in mangosteen (*Garcinia mangostana* L.)

Discriminating pairs of age groups		Magnitude of the values			Total number of positive values
Age group I	Age group II	Fruit index	Seed index	Seedling index	
1	2	+	-	-	1
1	3	-	-	+	1
1	4	-	+	+	2
Total positive values for age group 1					4
2	1	-	+	+	2
2	3	-	+	+	2
2	4	-	+	+	2
Total positive values for age group 2					6
3	1	+	+	-	2
3	2	+	-	-	1
3	4	+	+	-	2
Total positive values for age group 3					5
4	1	+	-	-	1
4	2	+	-	-	1
4	3	-	-	+	1
Total positive values for age group 4					3

Table 8 - Criteria and criterion for discrimination of age groups in mangosteen (*Garcinia mangostana* L.)

Sl. No	Age groups comparisons	Criteria 1	Criteria 2	Criterion	Conclusion
1	<25 and 25-50	-4.41	-4.84	-4.62	If the value is >-4.62 it falls in Group I, if not Group II
2	<25 and 51-75	-6. 19	-8.57	-7.38	If the value is >-7.38 it falls in Group I, if not Group II
3	<25 and >75	4.26	2.52	3.39	If the value is > 3.39 it falls in Group I, if not Group II
4	25-50 and 5 1-75	8.96	6.17	7.57	If the value is > 7.57 it falls in Group I, if not Group II
5	25-50 and >7 5	30.17	19.87	25.02	If the value is >25 .02 it falls in Group I, if not Group II
6	51-75 and >75	18.47	13.61	16.04	If the value is >16.04 it falls in Group I, if not Group II

For group 25-50 years

$Y = -0.0562\ FI + 0.0129\ SI + 0.9979\ SDLI + 4.331$ (adj. $R^2 = 0.99$)

For the 51-75 years group

$Y = -0.0479\ FI + 0.0115\ SI + 0.9967\ SDLI + 3.070$ (adj. $R^2 = 0.99$)

For the more than 75 years group

$Y = -0.0495\ FI + 0.0191\ SI + 0.9955\ SDLI + 2.247$ (adj. $R^2 = 0.99$)

For the average of all age groups

$Y = -0.0527\ FI + 0.0446\ SI + 0.9978\ SDLI + 1.056$ (adj. $R^2 = 0.99$)

Where FI- fruit index, SI- seed index and SDLI- seedling index.

The average of fruit index, seed index and seedling index values for each age group were fitted in the equation independently and the selection index values were determined. The averages of all the age groups were recorded and are given in Table 9.

Table 9 - Mean selection index or total index values of four age groups in mangosteen (*Garcinia mangostana* L.)

Age groups (Years)	Selection index values
Less than 25 years	93.38
25 - 50 years	101 .45
51 - 75 years	62.66
More than 75 years	64.84
Mean	80.64

Index values

The highest index value was found in the 25-50 years age group of mother plant, which was concluded to be the best age group for selection of the mother plants. This was followed by the age group of less than 25 years. The mean values of the important characters in the best age group (25-50 years) were fixed on the basis of the corresponding character for selection. Classification was made as above average if positively correlated and below average, if negatively correlated.

4. Discussion and Conclusions

Presentation of the results of the studies are broadly discussed and organized under subheadings to make them more accessible to the reader.

Association of morphological characters of fruit, seed and seedling

The fruit index was positively correlated with most of the fruit and seed characters, except fruit specific gravity, number of one-seeded fruits, seed thickness at centre, seed specific gravity, number of seedlings per fruit, and germination rate; all the seedling characters were negatively correlated. An analysis of the age group variations revealed that the maximum values of all the morphological characters of fruit and seed were recorded in the 51-75 years group and the lowest values were recorded in the 25-50 years group. As fruit index is a function, which is derived from all the morphological characters of fruit, the characters ought to be naturally correlated. However the negative correlations observed were due to the lowest values of fruit characters recorded in the 25-50 years age group, which in turn gave maximum seeded fruits, maximum germination of seeds, maximum survival rate and the best quality seedlings as revealed by the improved morphological characters.

Seed index was observed to be negatively correlated with all the fruit characters. Although the pattern of gradation observed in the values of seed characters in the various age groups was the same as that observed for fruit characters in the same age group, a corresponding size or weight of the seed was not observed. The relative differences observed at seed level are not as explicit as in the case of fruits and this could be the reason for the negative correlation observed between seed index and fruit characters. This is more evidenced if a ratio of the seed to fruit weight is computed. The second age group registered the highest ratio values, which again reveals that seed weight expressed as a fraction of the fruit weight is a more important character. Similarly, the differences are most clear in seed thickness to fruit girth ratio, where the second age group showed a very high ratio in spite of the fact that for both of the above characters the first age group had the highest values individually.

The positive correlations of seed index with all seedling characters once again confirms that the seedling characters are more governed by both the individual as well as collective characters of the seed. While in the standard methodology that was adopted, equal importance (weightage) was given to seed, fruit, and seedling characters and age of the mother plant, the results confirm that it is the seed characters which play a more determining role for better seedling characters. Even though identical reports are not available in mangosteen, the studies of Reddy (1997) revealed that the size of the seeds was highly variable, the difference in seed weight brought about the variation in germination and that the ability to germinate and grow successfully is related to the amount of food stored in the seed. Another author suggested that it is better to establish new plantings only from the larger seeds weighing 1 g and above in Florida (Campbell, 1966).

Seedling index, which was computed as a total variation of all the seedling characters collectively, was negatively correlated with number of seedlings per seed, number of days to germination, and seeds producing more than two seedlings. This is basically due to the fact that as a consequence of an increase in the number of seedlings per seed, the growth characters of the seedling are affected. The number of days taken to germinate was negatively correlated with seedling index because the seed characters

were positively correlated with seedling characters and the age groups that recorded the highest values for seed morphological characters also recorded the least number of days to germination. The number of seeds producing more than two seedlings were negatively correlated with the seedling index as the positive aspect of more seedlings produced per seed was negated by the character of comparatively slower growth. Another positive aspect in this case is that normally seedlings with exposed food storage or haustorial cotyledons are very vulnerable, and this attractive food source is prone to attack by rodents when it is present above ground. However in the Garcinia type, the food reserve is stored in the hypocotyl, which is protected by the envelopments and situated at, or below, soil level making it less vulnerable (Vogel, 1980).

In general, most of the fruit characters are positively correlated with seed characters and negatively correlated with seedling characters; most seed characters are positively correlated with seedling characters. Generally this is because the values of fruit characters increase with mother plant age group, but this increase is not matched by a corresponding increase in seed characters. In the case of seedlings, the age groups with increased fruit characters produce only weaker seedlings, as revealed by the lower seedling index. The increase generally observed in the seed characters leads to increased seedling growth and, hence, over emphasizes the absolute command of seed characters in determining the quality attributes of seedlings.

Principal component analysis. Fruit characters and index

A figurative plot of the principal components of the fruit characters is presented in figure 6, showing very narrow angles among the fruit characters such as rind weight, pulp weight, fruit weight, fruit index, number of segments, fruit girth and fruit volume and describing actually the high positive correlation that exist between them. The principal component analysis of fruit characters and age of the mother plant showed that factor I and factor II account for 88.76% of the cumulative variance. A critical analysis of the plot reveals that for all major characters of the fruit, age group three showed narrow angles, underlining that most of the improved fruit morphological characters are observed in this group. On the other hand, seedlessness showed narrow angles to age groups three and four, confirming that it is a factor that goes hand in hand with increased age. Seedlessness in fruits and seediness (one-seeded, two-seeded and more than three-seeded) are in opposite directions, illustrating that they are negatively correlated. A similar positioning is observed in the case of specific gravity and pulp weight, which reconfirms the negative correlation.

Seed characters and index

Principal component analysis of the seed characters and age of the mother plant showed that factors I and II account for 92-94% of the cumulative variance. A critical perusal of the plot (Fig. 3) of the seed characters revealed that maximum seed characters (seed index, selection index, germination percentage, seed producing one seedling, number of seedlings per seed and seed thickness at centre) were in close proximity and with very narrow angles to age group two, which clearly establishes the distinct superiority of this age group. The number of ungerminated seeds was in close proximity to age group four and positioned opposite germination percentage, showing the negative correlation. Similarly, seed weight and volume are positioned at opposite ends with respect to the days to germination and specific gravity, confirming the highly significant negative correlation observed in the study.

Seedling characters and index

Principal component analysis of the fruit characters and age of the mother plant showed that factors I and II account for 92.49% of the cumulative variance. The most important or major characters of the seedling and selection index are in close proximity to age group two (Fig. 4), which clearly establishes that seedlings of this age group are superior in all characters. The narrow angles between the various characters and this age group also establish the high positive correlations of the characters with this age group. The placement of survival rate and root to shoot dry weight ratio at opposite ends not only reveals the negative correlation but, more notably, the importance of roots which are critical in the case of mangosteen.

Step-wise regression

Step-wise regression was carried out to identify the variables contributing maximum variations and to reduce the number of variables. There was reduction in the number of variables influencing the fruit characters. With regard to seed characters, the thickness of the seed at the centre was found to be the most important factor. This is supported by the highest seed specific gravity found to be maximum in the best age group. For seedling index, the important characters were height of seedling, total number of leaves, total leaf area, survival rate, shoot and root fresh weight and dry weight, root length and total number of roots. As for seedlings, root characters were logically found to be important as the root system in mangosteen is magnolioid but cannot be recommended as an index for selection as they are underground. Mangosteen plants are very sensitive and even removal of bits of leaves for chemical analysis normally resulted in death of the plant.

Similarities between age groups based on fruit, seed and seedling characters

A critical analysis of the various age groups points to a very important conclusion: for most of the fruit characters, age groups one and two gave comparatively lesser values, whereas the older groups gave maximum values and have higher fruit index. Likewise in the case of seeds, the older groups had better size but the younger age groups gave more seeded fruits and gave better germination and more

seedlings per seed. These characters though were highest in the age group two were equally high in the youngest age group and thus the similarity between these two younger age groups were higher resulting in more closer distance in the dendrogram showing hierarchical clusters based on the Euclidean distance. In the case of seedling characters, a comparative study reveals more similarity and also when all fruit, seed and seedling characters were collectively taken. There was more similarity between the younger two age groups and this should be the reason for the very close distance between these age groups (less than 25 years and 25 - 50 years) and the close proximity in the dendrogram (Fig. 5).

Discriminant function

Further extrapolation of the coefficients (Table 7) used in discrimination of various groups were critically analysed and are presented in Table 8. The directions of coefficients were used to prepare the dendrogram presented in figure 7, showing both the positive and negative direction of the coefficients of fruit index, seed index and seedling index. It is again obvious from the table and the dendrogram that maximum coefficients were positively linked in the case of age group two when two specific pairs of age groups were discriminated as envisaged in this study. This was actually a reflection of the higher seed and seedling characters in age group two, compared to the other age groups, which reconfirms that this group is the best age group for selection as mother plants.

Selection index

Based upon the equation, the elaborated selection index revealed that the 25-50 years age group was the best. This is actually more a reflection of the improved seedling characters, particularly the germination percentage, survival rate and other morphological characters. The seed characters as revealed by the seed index were also highest in this particular age group. In the selection index equation, the seed characters were given equal weightage with all characters and hence this age group, which showed better seed characters, gave higher index values. Although the fruit characters were better in the older groups, seedlessness, low germination percentage and poor survival rate were the main reasons for the lower selection index values. Root characters cannot be taken as indices for selection as they are below ground, and hence not visible. Furthermore, any attempt to lift the bare seedlings normally results in death of the plants, as they are highly sensitive. As the root characters are positively correlated with shoot characters, selection based on the shoot characters reflects root characters as well.

Having identified the best age group, the mid value of the dominant characters were chosen as viable indices for the selection. The mid values of seedling characters were fixed so that destructive procedures could be avoided. Considering the complex of results obtained in the study, the following characters and mid values were selected as indices for selection.

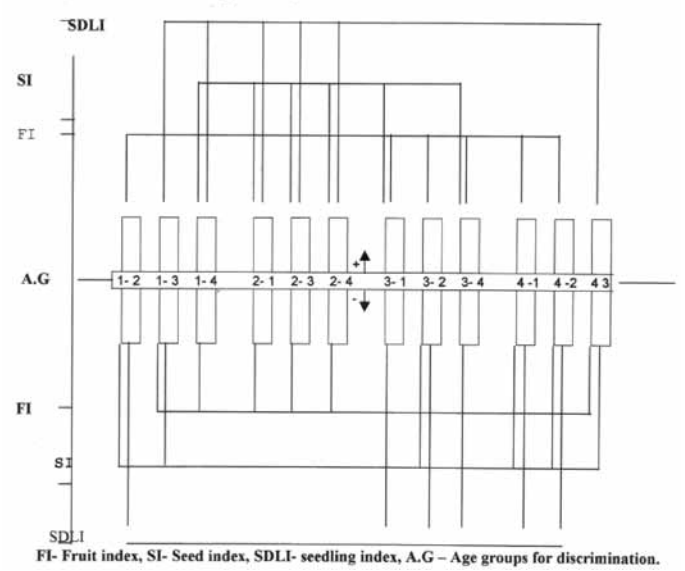

Fig. 7 - Dendrogram showing the directions of discriminant coefficients of fruit, seed and seedling indices of each age group when compared to the remaining three age groups in mangosteen (*Garcinia mangostana* L.).

The selection should be such that the fruits from mother plants of the age group of 25-50 years should have fruit weight of not less than 58 g, fruit girth of not less than 5 cm, fruit volume of not less than 51 ml, pulp weight of not less than 16 g, rind weight of not less than 41 g, and number of segments less than 5.

Seeds obtained from the fruits with the above characters should have a seed weight of not less than 0.66 g, seed length of not less than 1.61 cm, seed thickness at centre of not less than 0.66 cm, seed volume of not less than 0.44 ml, seed specific gravity of not less than 1.50, days to germination not less than 21, germination percentage not less than 87% and preferably have more than one seed per fruit.

Furthermore, seedlings at the one-year stage from this age group with the identified fruit and seed characters should have a height of not less than 10 cm, seedling girth at collar region of not less than 2 cm, total number of leaves per seedling not less than 9, number of new flushes per year more than one, total leaf area per seedling not less than 72 cm^2 and survival rate not less than 85%.

In conclusion, it can be said that the present study has generated results of immense practical relevance which will directly aid in selection or act as a powerful tool for the selection of mother plants, fruit, seed and seedling characters for improved seedling growth.

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In vitro drought effects on morphological and physiological indices of two fig (*Ficus carica* L.) cultivars

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Key words: drought, growth parameters, polyethylene glycol, proline.

Abstract*: In vitro* responses of two fig cultivars, 'Sabz' and 'Siah', were evaluated in MS media containing four levels of polyethylene glycol (PEG) (0, 2, 4, 6%) as a simulation of water stress. The results showed that in Sabz cultivar, shoot length, shoot fresh and dry weights were 43, 36 and 25%, respectively, lower than control in drought treatments caused by 4% PEG, while the leaf area and specific leaf area were not significantly (P>0.05) affected. In Siah cultivar, shoot length, fresh and dry weights were 57, 58 and 40%, respectively, lower in stressed media in comparison to control. In contrast to Sabz cultivar, leaf area and specific leaf area of Siah cultivar were significantly reduced by addition of 6% PEG (56 and 21.5%, respectively). Naturally, the amount of proline in 'Sabz' was higher than in 'Siah' (81.8 µmole g^{-1} versus 16.7 µmole g^{-1}). However, in both cultivars, with addition of PEG in culture media, leaf proline content was increased, in comparison to control. With increasing PEG% in culture media, the amount of leaf soluble sugars content increased and the amount of starch decreased. The result show that 'Siah' is more sensitive to drought than 'Sabz' and that *in vitro* culture can be used to evaluate drought tolerance of cultivars.

1. Introduction

Despite considerable advances in technology, agriculture is exposed to climate changes in all parts of the world. Among climatic factors, rainfall is the most critical because 70% of the main areas under cultivation are still without irrigation (Wilhite, 2001).

Water shortage is the main characteristic of agriculture in Mediterranean regions, inducing water stress during spring and summer. Fruit trees survive in this situation because they are prone to physiological or morphological changes which enable them to avoid drying damage, cast it back, or tolerate it (Torrecillas *et al*., 1999). Drought tolerance is observed with different rates in almost all plant species. Understanding plant responses to the external environment is an essential component for selection stress tolerance (Reddy *et al*., 2004). In recent years, in southern Iran, low and poor distribution of rainfall has caused great damage to plants. Water stress, particularly in rain-fed fig production areas (e.g. Estahban) has become a big issue. In the Estahban region not only the annual production has decreased, but also the highly productive trees are in danger of destruction.

One strategy to confront this problem is the identification and selection of more tolerant genotypes. Traditional breeding approaches for selection of tolerant plants are time consuming and complex processes. Imposing water stress in field-grown crops is difficult because of the unpredictability of rainfall and the possibility of seepage from adjoining plots and drought escape of deep roots from osmotic stress. Since field evaluation of drought effects is highly correlated with environmental conditions, *in vitro* screening techniques allow for a better control of culture conditions. Tissue culture also offers the possibility of screening many plants in limited space and time, assuming that there is a correlation between cellular, tissue, organ and *in vivo* plant responses (Mohammad *et al*., 2000).

Polyethylene glycol (PEG) has been used to simulate water stress in plants. PEG of high molecular weight is a non-ionic osmoticum lowering the water potential of nutrient solution without being taken up or being phytotoxic (Hassan *et al*., 2004). It has been shown that the shoot length decreases with increasing water stress via *in vitro* culture in cherry (Sivritepe *et al*., 2008) and mulberry (Tewary *et al*., 2000). In a study on mulberry, leaf relative water content (RWC) decreased with increasing water stress (Ramanjulu *et al*., 1998).

Accumulation of proline is the most common plant response to decreasing water potential (Helal Ragab and Samir Moustafa, 2008). It has been reported that with increasing PEG in date palm seedling culture, the rate of proline was increased in tolerant cultivar (Djibril *et al*., 2005).

Carbohydrates have an important role in osmotic regulation in different plant parts (Masoudi-Sadaghiani *et al*., 2011). Simple sugars such as glucose and fructose were increased and the rate of sucrose and starch decreased under water stress (Sharp and Davies, 1979; Munns and Weir, 1981; Wang and Stutte, 1992; Nawar and Ezz, 1993; Clif-

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ford *et al*., 1998; Perez-Perez *et al*., 2007; Mafakheri *et al*., 2010). In Iran, Fars province with about 36,000 hectares under fig cultivation is the primary fig production area. Estahban district with 20,000 hectares under fig cultivation is the center of fig cultivation in Fars province, as well as in Iran. As mentioned above, due to drought in recent years, highly productive trees are in danger of destruction. The cultivars Sabz and Siah are the most desirable cultivars in Iran; there has not been report on water stress tolerance of these cultivars. Selection of more drought tolerant cultivars can be used to establish of new orchards and a promising rootstock for other interested cultivars.

The aim of this investigation was to evaluate the effects of drought created by polyethylene glycol (PEG) on morpho-physiological changes of two important fig cultivars ('Sabz' and 'Siah') and leading to selection of more drought tolerant varieties using *in vitro* culture.

2. Materials and Methods

Plant material and explant decontamination

In this research, the *in vitro*-derived micro-shoots of two cultivars of *Ficus carica* L. 'Sabz' and 'Siah' were used. The branches (20~30 cm long) were cut from rain-fed, mature mother trees established in Estanban Fig Research Station located at 29° 07' N latitude and 54° 02' E , 197 km southeast of Shiraz. Freshly grown stems with 2~6 nodes were cut from the above branches and washed in a solution of water-detergent (three drops of detergent in 100 ml of water) for 30 min, then placed in a solution of benomyl (3%) for 1 h (Torres, 1998). To prevent the secretion of phenolic compounds into the culture media, explants were treated in a solution of 2% ascorbic and citric acid for 45 min. At this point, the stems were transferred to a laminar air flow cabinet. They were first dipped in 70% (V/V) ethanol for 45 s, then treated with 15% (V/V) Chlorox solution (a household bleach containing 5.25% sodium hypochlorite) for 15 min and rinsed three times with sterilized distilled water. Nodal segments of 1~1.5 cm long were cut and used as explants. The basal medium was MS (Murashige and Skoog, 1962), containing 3% sucrose (MERCK, LGaA 64271Darmstadt, Germany) and 1mg l^{-1} (4.4 µM) benzyl adenine (BA) and solidified by 0.8% agar-agar (MERCK, LGaA 64271Darmstadt, Germany). The pH was adjusted to 5.7±0.05 prior to autoclaving at 1.2 atm pressure and 121˚C for 20 min. Cultures were maintained in a growth chamber at 25±2˚C, 58% relative humidity and a photon flux density of 40 µmol m^{-2} s^{-1} was provided by white fluorescent tubes, with a 16 h photoperiod.

PEG treatments

After four weeks, the micro-shoots (2 cm length) from the above cultures were transferred into 500 ml culture vessels containing 100 ml MS basal media supplemented with different levels of PEG (0, 2, 4 and 6%) as selective agent and maintained under the environmental conditions described above.

Growth measurements

Seven weeks after the beginning of experiments, shoot length and shoot fresh and dry weight were evaluated.

Leaf area was measured by leaf area meter (Delta-Tdevices England) and specific leaf area was calculated as follows:

specific leaf area = leaf area (cm^2)/leaf dry weight (g)

Relative water content

Leaf relative water content (RWC) was estimated according to the method described by Whetherley (1950). Twenty healthy leaf discs of 0.7 cm diameter were cut from plants, using a leaf punch and washed three times with double distilled water. Leaf discs were weighed (FW), then placed into a 10 ml conical flask, immersed in 10 ml distilled water for 4 h in dark. Turgid weight (TW) of leaf discs were then measured and samples were dried in an oven (80ºC) until constant weight (DW) was achieved. RWC was calculated from the following equation:

Relative water content % = FW - DW/TW - DW x 100

Chlorophyll content

For chlorophyll determination, prior to extraction fresh leaf samples were washed with deionized water to remove any surface contamination. One g leaf samples were ground in 80% acetone using a pestle and mortar. The mixture was centrifuged at 4800 rpm for 20 min. The optical density of the supernant was measured at 663 and 645 nm wavelengths and chlorophyll content was calculated using the following equation:

Mg Chl g FW = [20.2 (OD645 nm) + 8.02 (od663 nm)] x V/FW x 1000

where V is final volume of solution (ml) and fw, leaf fresh weight (mg).

Proline contents

To determine proline content of the leaves, 0.5 g of plant material was homogenized in 10 ml of 3% aqueous of sulfosalcylic acid and the homogenate filtered through Whatman # 2 filter paper. Two ml of filterate was reacted with 2 ml acid ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed for 15~20 seconds. Chromophore containing toluene was aspirated from the aqueous phase and the absorbance was read at 520 nm. The proline concentration was determined based on standard curve and calculated as follows:

$$\mu \text{ moles proline/g FW} = \frac{(\mu g \text{ proline/ml x ml toluene})}{115.5 \ \mu g / \mu mole} \Big/ \text{g sample/5}$$

Electrolyte leakage

Electrolyte leakage was measured as an assessment of cell wall permeability. Electrolyte leakage was measured using an Electrical Conductivity Meter. Two mature leaves per plant were taken and cut into 1 cm segments. After three washes to remove surface contamination, leaf samples were placed in individual vials containing 10 ml of

distilled water. The samples were incubated at room temperature on a shaker (100 rpm) for 24 h. Electrical Conductivity (EC) of bathing solution (EC1) was read after incubation. Samples were then placed in an autoclave at 120°C for 20 min and the second reading (EC2) was determined after cooling the solution to room temperature. The electrolyte leakage was calculated as EC1/EC2 and expressed as percent.

Soluble sugars content

Soluble sugars were extracted from 0.1 g fresh leaves by heating with 5 ml of 80% ethanol in a water bath at 70°C for 30 min. The insoluble residue was removed by centrifuging at 5000 g for 10 min. One ml of the resulting extract was mixed with 1 ml of 5% phenol solution and 5 ml of sulfuric acid. The mixed solution was permitted to cool to room temperature, then vortexed. The absorbance was read at 490 nm using a spectrophotometer. The soluble sugar content of each sample was determined using standard curve for glucose and expressed as mg glucose g^{-1} FW (McCready *et al*., 1950; DuBois *et al*., 1956).

Starch content

The solid residue remaining in the centrifuge tube after removal of all soluble sugars in the previous section was washed, re-extracted and re-centrifuged four times using 80% (V/V) ethanol. Starch content in the samples was determined colorimetrically using anthrone method (McCready *et al*., 1950). The absorbance was determined at 630 nm in a digital spectrophotometer (Spectronic 20 D+; Spectronic Instruments Inc., New York, USA.) as described by López *et al*. (2002).

Statistical analysis

The experiment was arranged as a 2×4 factorial experiment in completely randomized design (CRD) with 10 replicates, each consisting of three plants. Thus, there were 30 plants in each treatment and a total of 240 plants in the experiment. Data were subjected to analysis of variance using the SPSS software (ver. 13.0) SPSS Inc. Mean differences were determined by Duncan's multiple range tests at $p \leq 0.05$.

3. Results and Discussion

Water stress caused a reduction in micro-shoot growth of the two fig cultivars (Table 1). In 'Sabz', shoot fresh and dry weights and shoot length were 36, 25, and 43% lower, respectively, in drought treatments caused by 4% PEG compared with the control, while in 'Siah', these traits were 58, 40 and 57% lower respectively, in the same stressed media (4% PEG), than the control. In this latter cultivar, in contrast to 'Sabz', leaf area and specific leaf area were significantly affected by water deficit (56 and 21.5%, respectively) (Table 2). The findings of this study showed that the growth rate of 'Siah' in optimum conditions (control) was the same as Sabz cultivar, but with increasing intensity of water stress, the growth reduction rate in 'Siah' was more than in 'Sabz'. The main effect of cultivars showed that without respect to different levels of PEG, 'Sabz' had a significantly higher leaf area (8.2 cm) than 'Siah' (5.51) (Table 2). Water stress significantly reduced vegetative growth indices of fig micro-shoots. This phenomenon has been previously reported (Oukabli *et al*., 2008).

Table 1 - Interaction of water stress and cultivar on micro-shoot length, fresh and dry weight

	Drought stress (PEG%)				
	0	2	4	6	
Cultivar	Average fresh weight (g)				Mean
Siah	3.80 a [z]	2.1 bc	1.6 c (58)	2.10 bc	2.4 A
Sabz	3.00 ab	2.3 bc	1.9 bc (36)	2.10 bc	2.3 A
Mean	3.41 A	2.2 B	1.8 B	2.08 B	
	Average dry weight (g)				
Siah	0.50 a	0.30 bc	0.3 bc (40)	0.30 bc	0.34 A
Sabz	0.40 ab	0.40 ab	0.3 bc (25)	0.30 bc	0.36 A
Mean	0.46 A	0.34 B	0.3 B	0.31 B	
	Shoot length (cm)				
Siah	2.60 a [z]	1.4 b	1.1 b (57)	1.20 b	1.57 A
Sabz	2.33 a	1.4 b	1.3 b (43)	1.30 b	1.59 A
Mean	2.47 A	1.4 B	1.2 B	1.25 B	

[z] In each row and column, means with similar letters (small letters for interaction and big letters for main effects) are not significantly different using Duncan's multiple rang test P≥0.05.
The percentage of reduction with respect to control is reported in parentheses.

Table 2 - Interaction of water stress and cultivar on leaf area (cm^2) and specific leaf area (g cm^{-2}) of micro shoots

	Drought stress (PEG%)				
	0	2	4	6	
Cultivar	Leaf area (cm^2)				Mean
Siah	8.2 a [z]	6.9 ab	3.4 b	3.6 b (56)	5.51 B
Sabz	12.4 a	8.2 a	6.9 ab	6.6 ab	8.52 A
Mean	10.0 A	7.6 AB	5.4 B	5.4 B	
	Specific leaf area (g cm^{-2})				
Siah	14.4 a	14.6 a	13.4 a	11.3 b (21.5)	13.4 A
Sabz	16.8 a	15.3 a	15.1 a	13.1 a	15.1 A
Mean	15.6 A	14.9 A	14.4 A	12.2 A	

[z] In each row and column, means with similar letters (small letters for interaction and big letters for main effects) are not significantly different using Duncan's multiple rang test P≥0.05.
The percentage of reduction with respect to control is reported in parentheses.

The long-term use of PEG *in vitro* on growth reduction and shoot regeneration in other plants has been well documented (Bressan *et al*., 1982; Handa *et al*., 1982; Handa *et al*., 1983; Dami and Hughes, 1995; Al-Khayri and Al-Bahrany, 2004). In most cases, PEG has been used to stimulate

water stress in plants. PEG with high molecular weight is a non-penetrating inert osmoticum which lowers the water potential of nutrient solutions without being taken up or being phytotoxic (Hassan *et al*., 2004). It has been shown that *in vitro* growth reduction of apple (Molassiotis *et al*., 2006) and cherry (Sivritepe *et al*., 2008) was due to the decrease in water potential created by PEG.

Under water stress conditions, the reduction of mineral absorption has resulted in limited leaf growth and development and decreased plant water transpiration. Therefore, producing smaller leaves can be the first plant defense mechanism against water deficit. Hsiao (1973) reported that a decrease in leaf area results in lower light absorption and photosynthetic capacity; thereby reducing photosynthetate and plant growth. This can be true with plants *in vivo*, but probably this is less important *in vitro*, because *in vitro* plants are more heterotrophic, so decreases in their growth cannot be due to a deficiency of carbohydrates. In the present work it was clearly shown that the decrease in water availability is the main cause of decreasing growth. Specific leaf area (SLA) is a function of leaf area. The reduction of SLA in water deficit conditions is due to the fact that leaf area development is more affected than deposition of dry matter (Blum and Pneul, 1990).

In both cultivars with increasing PEG% in culture media, leaf relative water content (RWC) decreased and an increase in ion leakage was recorded (Table 3, Fig. 1). These findings are in agreement with the results obtained by other researchers *in vitro* (Sawwan *et al*., 2000; Al-Khayri and Al-Bahrany, 2004; Chai *et al*., 2005). Leaf yellowing and chlorosis were the consequence of structural damage to cell membranes in explants. Interestingly, in 6% PEG, although the rate of relative water content reduction in leaves of Sabz cultivar (50%) was more than 'Siah' (20%), the difference in their ion leakage was not significant.

Leaves accumulated significant quantities of proline and, in contrast, the amount of chlorophyll was decreased with increasing PEG percentage in culture media (Fig. 2).

Table 3 - Interaction of water stress and cultivar on leaf relative water content (%) and leaf ion leakage (%)

	Drought stress (PEG%)				
	Leaf relative water content (%)				
Cultivar	0	2	4	6	Mean
Siah	69.2 a [z]	68.6 a	59.3 ab	54.9 ab (20)	63.0 A
Sabz	73.6 a	51.4 ab	61.7 a	36.7 b (50)	55.8 A
Mean	71.4 A	60.6 AB	58.8 AB	45.7 B	
	Leaf ion leakage (%)				
Siah	50.6 d	57.1 bcd	55.5 bcd	64.2 abc	56.9 B
Sabz	54.1 cd	57.0 bcd	68.7 a	66.2 a	61.5 A
Mean	52.3 C	57.0 BC	62.1 AB	65.2 A	

[z] In each row and column, means with similar letters are not significantly different using Duncan's multiple rang test P≥0.05.
The percentage of reduction with respect to control is reported in parentheses.

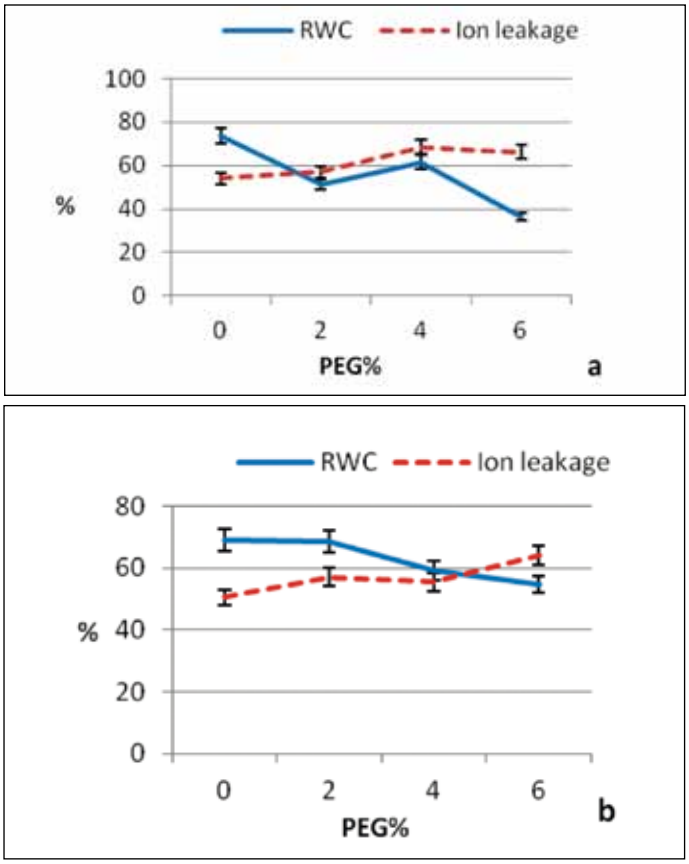

Fig. 1 - Changes in leaf water content (RWC) and ion leakage in two fig cultivars: 'Sabz' (a) and 'Siah' (b).

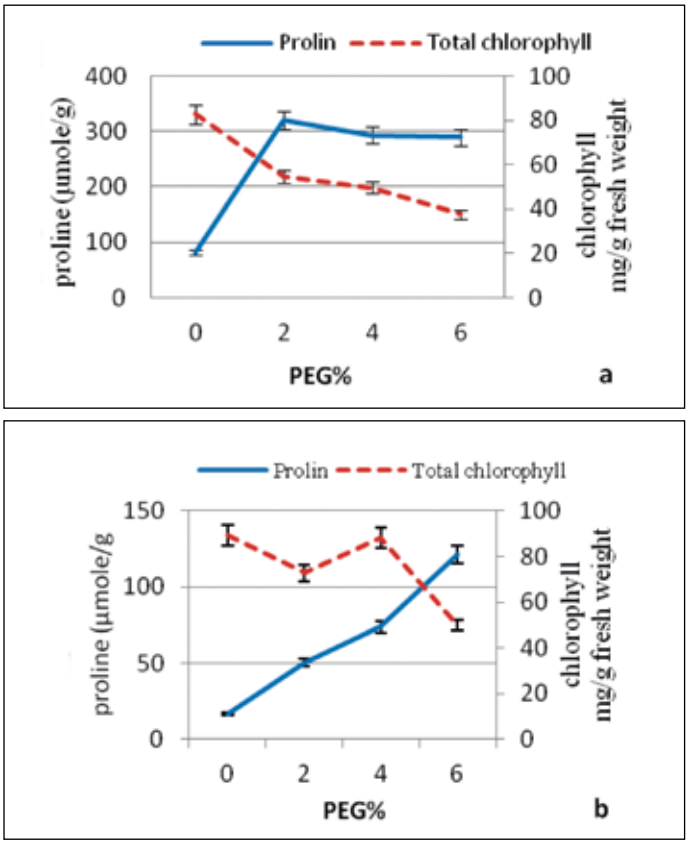

Fig. 2 - Changes in proline and total chlorophyll in two fig cultivars: Sabz (a) and Siah (b).

Naturally, the amount of proline in 'Sabz' was higher than in 'Siah' (81.8 µmole g^{-1} versus 16.7 µmole g^{-1}) (Table 4). In both cultivars, with increasing PEG % in media, proline content increased. However, the results indicated that, in all levels of PEG treatments, the amount of proline in 'Sabz' was higher than in 'Siah'. Plants produce and accumulate such compounds compatible to their metabolism to overcome adverse effects of drought stress (Zhu, 2001). Accumulation of such substances would cause more negative water potential in plants, a necessary condition to absorb and keep the water in plant tissues (Sivritepe *et al*., 2008).

Table 4 - Interaction of water stress and cultivars on leaf proline content (µm g^{-1} fresh weight)

	Drought stress (PEG%)				
Cultivar	0	2	4	6	Mean
Siah	16.8 c [z]	50.5 b	73.7 b	121.0 b	65.5 B
Sabz	81.8 b	320.7 a	293.6 a	289.5 a	246.4 A
Mean	42.8 B	170.6 A	199.4 A	205.2 A	

[z] In each row and column, means with similar letters are not significantly different using Duncan's multiple rang test P≥0.05

With increasing the severity of water stress, proline and soluble sugars (as compatible substances) accumulated significantly in explant leaves of both cultivars. The accumulation of these substances has been well documented in field (Clifford *et al*., 1998; Zamani *et al*., 2002; Mafakheri *et al*., 2010) and *in vitro* conditions (Handa *et al*., 1982; Brito *et al*., 2002; Al-Khayri and Al-Bahrany, 2004; Molassiotis *et al*., 2006; Sivritepe *et al*., 2008). A notable point in our results was the significant increase in proline accumulation in leaves of both cultivars. It has been reported that there is a direct positive relationship between drought tolerance and proline concentration in plant tissues (Al-Khayri and Al-Bahrany, 2004; Sivritepe *et al*., 2008). The role of proline in destroying reactive oxygen species (ROS) in water stress conditions has been reported in different plant species (Turkan *et al*., 2005; Verslues *et al*., 2006). Therefore, it can be expected that plants which accumulate more proline under water stress are more tolerant. Thus, it may be concluded that 'Sabz' is more tolerant than 'Siah', although in some plants a relationship was not found between proline accumulation and drought tolerance.

The changes in leaf sugar and starch contents under different levels of PEG are shown in Table 5 and figure 3. In both cultivars, with increasing PEG%, the amount of soluble sugar content increased and the amount of starch deceased. In drought conditions, soluble sugar can act as osmoticum and also osmoprotectant. In addition, accumulation of sugar may partly protect protein against the oxidative damage created by free radicals (Bohnert and Shen, 1999). In the present work, soluble sugars content in 'Sabz' was greater than in 'Siah', but it did not have significant effect on water absorption. Accumulating soluble sugars can be due to starch hydrolysis, which is in agreement with the results obtained by Shawky *et al*. (1997). Taylor *et al*. (1982) also reported that carbohydrates (reducing and non-reducing sugars) are the most abundant component in osmotic adjustment in tomato seedlings.

Table 5 - Interaction of water stress and cultivar on leaf TSS and starch (mg g^{-1} dry weight)

	Drought stress (PEG%)				
	0	2	4	6	
Cultivar	TSS (mg g^{-1} dry weight)				Mean
Siah	170.3 c	168.9 c	256.6 bc	287.0 b	220.69 B
Sabz	276.4 bc	344.5 b	357.5 b	522.2 a	375.2 A
Mean	215.8 C	256.7 BC	311.6 B	404.6 A	
	Starch (mg g^{-1} dry weight)				
Siah	133.5 ab	103.8 bc	73.7 bc	65.3c	94.1 A
Sabz	167.0 a	124.2 abc	76.3 bc	80.3 bc	111.9 A
Mean	152.56 A	112.83 B	75.4 B	73.9 B	

[z] In each row and column, means with similar letters (small letters for interaction and big letters for main effects) are not significantly different using Duncan's multiple rang test P≥0.05

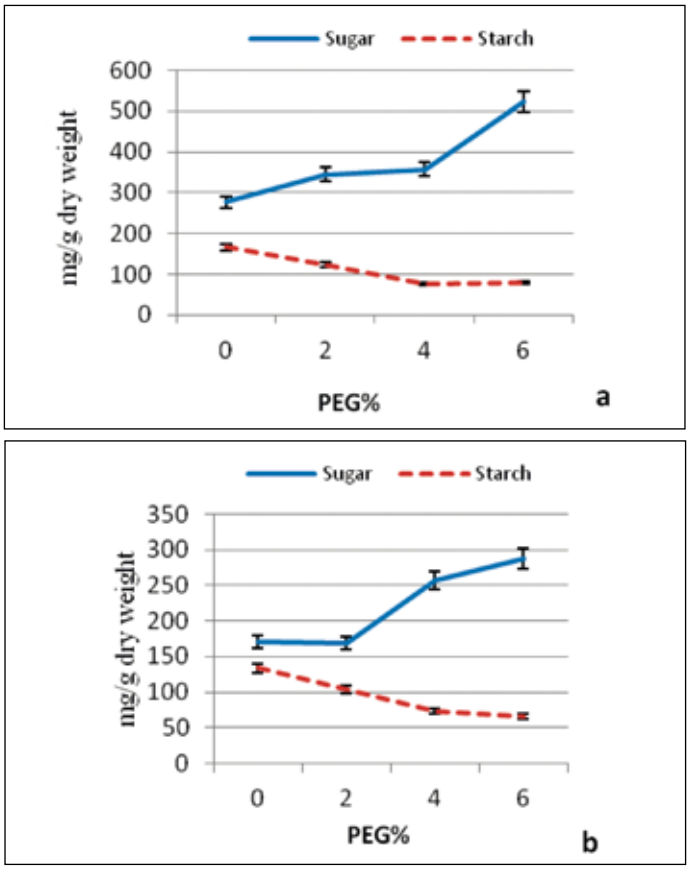

Fig. 3 - Changes in leaf sugar and starch content in two fig cultivars: 'Sabz' (a) and 'Siah' (b).

In this study, with increasing PEG in culture media, the amount of total chlorophyll declined in leaves of the two fig cultivars. Effect of drought stress on reduction of chlorophyll content of other plants *in vitro* has previously

been reported (Hernández-Sebastià *et al*., 2000; Brito *et al*., 2002; Molassiotis *et al*., 2006). Under field conditions, also chlorophyll content was reduced with increasing drought (Munne-Bosch and Penuelas, 2004). In the present experiment, with increasing water deficit (with addition of PEG), chlorophyll content was decreased along with accumulation of leaf proline (Fig. 3). This may be due to the fact that chlorophyll and proline are synthesized through glutamate pathway, causing an increase in synthesis of proline under drought conditions, which resulted in reduction of chlorophyll synthesis (Aspinall and Paleg, 1981).

4. Conclusions

The assessment of drought tolerance of two fig cultivars showed that under water stress conditions in both cultivars, leaf fresh and dry weight, total chlorophyll and starch contents decreased, but proline and soluble sugar increased. Finding of this research showed that Siah cultivar was more vigorous than Sabz cultivar in control treatment, even if the differences were not statically significant. In stress conditions, the growth rate reduction in 'Siah', was more obvious than in 'Sabz', and this latter cultivar was more drought tolerant, possibly due to accumulation of proline and soluble sugars. The results of our work show that it is possible to use *in vitro* culture as a successful method for selection of tolerant varieties.

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Quantitative analysis of soil water content in young drip-irrigated olive orchards

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Key words: evapotranspiration, neutron probe calibration, root density, stage of development.

Abstract: The present work was carried out in northern Tunisia (36°40' N, 10°16' E) during a single growing season in order to examine how water is distributed within young drip-irrigated olive orchards on the basis of distance to trunk and depth. Soil water content was measured by using a time domain reflectometer (TDR) and a neutron probe calibrated by concurrently measuring soil water content gravimetrically. Measurements were made below the canopies, along the line of drippers and out of the projected canopy area, at distances of 1.4 m, 2.2 m, 2.8 m and 4.2 m from trunks (Compartments G_1, G_2, G_3 and G_4), taking into account the heterogeneous distribution of roots. Results showed significant and positive correlations between the series of data collected simultaneously with the PVC and aluminium access tubes and those collected using the different methods and apparatus, demonstrating that any device may be employed, depending only on their availability. Results showed significant changes in soil water contents and stocks according to the season, depth and distance to trunk. During the rainy period, the stocks of water increased homogeneously within all soil compartments, but varied consistently during the dry season with lower values recorded within the upper soil layers. The area situated at 4.2 m from trunks was the driest in summer but it was the wettest during the rainy period. No roots were found at this distance while maximum root densities were observed at 0.4 m from trunk within the upper layers. The lack of water recorded after June affected tree height and fruit growth rates, although irrigation application was sufficient to meet the seasonal crop water needs.

1. Introduction

In Tunisia water resources (36 Km^3 annually) are mostly used to irrigate about 400 000 ha of annual and perennial crops. These amounts fulfill on average 75% of the crop water requirements (Hamza, 2009). In the north and center of the country, priority is always given to fruit trees and vegetables, although olive is considered the most important species and it is cultivated over a large area (1 700 000 ha).

Over the two last decades, the amount of available water has decreased consistently, thus the imbalance between water supply and water demand has intensified. This situation has given rise to much attention from the relevant authorities and the general public in terms of the average and long term water uses. Obviously, water should be used judiciously with reasonable amounts to meet water needs and without any wastage.

Determination of crop water needs, i.e. crop evapotranspiration (ETc), is therefore necessary to efficiently manage irrigation at the field level. However, it involves a highly complex set of processes which are influenced by watering conditions and tree and land cover characteristics. We have published in recent years technical papers (Masmoudi-Charfi, 2006; Masmoudi-Charfi *et al*., 2006) presenting the water requirements of olive trees for different cultivation areas, according to age, soil coverage and growth stages based on the climatic method of the FAO (Allen *et al*., 1998; Habaieb and Masmoudi-Charfi, 2003; Masmoudi-Charfi *et al*., 2004). However, during the calculation procedure, we were confronted with a lack of information about the crop coefficient. In addition, long term climatic data were not available for all sites.

The lysimetric measurements give more precise information on water use, but is hard to carry out and expensive (Deidda *et al*., 1990).

Estimates of actual evapotranspiration for adult olive trees were published in Tunisia and elsewhere for different environments (Ozyilmaz and Ozkara, 1989; Cohen, 1991; Pastor *et al*., 1998; Michelakis, 2000; Musters and Bouten, 2000; Palomo *et al*., 2002; Bandino and Dettori, 2003). These estimates require regular measurements of soil water content, which are essential to calibrate models estimating the vertical distribution of root water uptakes (Hazrat *et al*., 2000; Palese *et al*., 2000). Gravimetry is amongst the devices used to reliably measure soil water content (Hv) in the field. However, it is more useful for calibrating other devices than for scheduling irrigation because it takes a full day to dry samples and irrigation may be needed before the results of the measurements are obtained. The time domain reflectometer (TDR) is easy to use and reliable but the number of sites for measurements is limited. The neutron-scattering method was extensively used in field studies for measuring soil storage and its changes over

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time (Vachaud *et al*., 1977; Evans *et al*., 1996; Tarara and Ham, 1997; Xiong and Guo, 1999). With this apparatus, measurements can be made at different depths and sites, but these 'measurements' represent a property of the soil that can be related to soil-water content and are, therefore, indirect estimates (Hewlett *et al*., 1964; Rana and Katerji, 2000). On the other hand, a survey of literature (Hewlett *et al*., 1964; Vachaud *et al*., 1977; Sinclair and Williams, 1979; Haverkamp *et al*., 1984; Vauclin *et al*., 1984; Villagra *et al*., 1995) shows that little attention has been paid to the associated errors and uncertainties resulting from the definition of the calibration curve itself, when calibrating the apparatus. Instrumentation, timing and location variances are identified as the different components of the total variance of an individual water content estimate. Sinclair and Williams (1979) reported a comprehensive analysis of the contribution of instrument calibration and location variances to the variance of mean water content values and their changes in time. Implicitly they assumed that all the observations were independent of one another.

The present work illustrates, with results from a single growing season of a young olive orchard cv. Chétoui aged six years and cultivated in northern Tunisia, how water is distributed in such orchards taking into account time (stage of development) and root distribution. Our approach is based on estimating water content at different distances from trunks. Data were analyzed considering both spatial and temporal variability within different soil reservoirs, throughout the campaign and on some typical days. Specifically, the aim was to highlight the main difficulties found when measuring soil water content in drip-irrigated orchards characterized by discontinuous and low soil coverage.

2. Materials and Methods

Experiment site

The study was performed during a single growing season (2003) on a young olive orchard located at the experimental farm of the Institut National Agronomique de Tunisie, northern Tunisia (36°40' N, 10°16' E). The area is characterized by a Mediterranean climate with average annual water deficit of 750 mm and reference evapotranspiration (ET0) of 1200 mm. Weather variables were recorded continuously in a nearby automatic weather station. Daily average values were used for ET0 calculation (Table 1) following the Penman Monteith equation (Allen *et al*., 1998).

Table 1 - Annual and seasonal (March - September) weather variables recorded in 2003

Weather variables	Value
Rainfall (mm/year)	790
Seasonal rainfall (mm)	346
ET_0 (mm/year)	1211
Seasonal ET_0 (mm)	982

The year of experiment was rainy and hot with annual and seasonal effective rainfall amounts of 546 mm and 239 mm, respectively. These values were estimated following the USDA method (FAO, 1976). Average maximum and minimum temperatures (24.9°C and 14.9°C, respectively) showed an increase of 3 and 7%, respectively, with regard to the average values recorded during the 25 previous years. Rising temperatures were noted during the three first months of the year, resulting in an increase of the growth degree day (GDD) of about 300 days.

Olive orchard

Three six-year-old olive trees (cv. Chétoui), representative of the whole orchard, were used in this experiment. They were planted at 6 m x 6 m spacing and stand on a textured clay-loamy soil of about 2 m depth. Soil characteristics were determined at the beginning of the experiment for each trench of soil to 1 m depth (Table 2). Average bulk density (d_a), soil water contents at field capacity (θ_{cc} measured at -0.3 MPa) and at wilting point (θ_{pfp} measured at -1.5 MPa) were 1.6 g/cm^3, 0.50 m^3/m^3 (50%) and 0.26 m^3/m^3 (26%), respectively.

Table 2 - Soil characteristics of the olive orchard

Horizon (cm)	0-20	20-40	40-60	60-80	80-100	Average	Ecartype
Clay %	39	34	28	22	20	29	7.1
Loam %	50	52	48	48	46	49	2.0
Sand %	11	14	24	32	34	23	9.3
d_a (g/cm^3)	1.55	1.64	1.60	1.60	1.68	1.61	0.04
θ_{cc} (%)	48	50	50	51	50	50	0.98
θ_{pfp} (%)	25	26	27	26	25	26	0.75

Trees were intentionally chosen of the same variety, with similar shape. Leaf area (LA) was determined after pruning by computing the number of leaves on representative branches and their specific area by planimetry (Fernandez and Moreno, 1999); individual average value of LA was 14 m^2. Soil coverage was low, rarely exceeding 35%. At the end of the campaign, mean tree height and canopy diameter reached 4.9 m and 4.0 m, respectively.

Water requirements and irrigation management

Average weather variables published in local papers (Masmoudi-Charfi, 2006) were used to estimate daily ET0 values. Crop evapotranspiration (ETc) was then determined following the FAO method for non-standard conditions (Allen *et al*., 1998) as $ET_c = ET_o \times K_c \times K_r$, with a crop coefficient $K_c = 0.5$ (six-year-old trees) and $Kr = 0.75$ (COI, 1997) accounting for an average soil coverage of about 33% (Masmoudi-Charfi, 2008).

Trees were irrigated from 15 May to 5 September with amounts ranging between 0.333 m^3/tree and 1.098 m^3/tree according to the stage of growth. The seasonal irrigation amount was 5.4 m^3/tree. Periods and doses of irrigation are

reported in Table 3. Water was supplied using four emitters per tree with a total discharge of 16 l/h. Fresh water was provided alternatively from the 'Medjerda' canal, the main river of northern Tunisia and nearby wells.

Table 3 - Irrigation supply periods and amounts (m^3/tree)

	1	2	3	4	5	6	7
Irrigation period	15-20/5	2-3/6	30/6-2/7	10-15/7	21-30/7	5-10/8	28/8-5/9
Irrigation amount	0.823	0.333	0.549	0.843	0.902	0.902	1.098

Field monitoring

Experimental protocol and soil water content measurements. This work was carried out in order to highlight the difficulties met at field level when elaborating protocols concerning irrigated olive orchards, characterized by heterogeneous distribution of light, soil coverage, roots and water application. Difficulties concerned mainly the choice of measurement sites and the right measuring device, particularly:

- At which depths, frequency and distances from trunks measurements should be taken?
- Which kind of apparatus and access tubes should be used for easy and precise soil moisture monitoring?
- Is there any relationship between measurements taken with different apparatus?
- How many repetitions (trees) are necessary to get significant results?
- What precautions should be taken when preparing and installing the access tubes and when calibrating the neutron probe?

Taking all these questions in mind, but also the results obtained for this same orchard relative to the root distribution (Masmoudi-Charfi and Ben Mechlia, 2011), a specific diagram was built in which the area surrounding the three olive trees was instrumented with access tubes covering all soil occupation cases. Figure 1 shows a series of 28 access tubes implemented vertically in the soil at distances from trunks ranging between 1.4 m and 4.2 m. Measurements of volumetric water contents (H_v,%) were carried out within this area from April to October at depths ranging from 0.20 to 1.20 m using a neutron probe (SOLO 25, Nardeux, France). Two types of tubes were experimented and compared. A correlation was then established between measurements made simultaneously with aluminum and PVC-polyamide tubes, which were locally assembled (4 cm inside diameter and 170 cm long). Specific glue was used to seal the components of the PVC-polyamide tubes in order to assure their tightness and impermeability to water. Neutron probe countings were coupled with routine observations of H_v made at the limit of the canopy (2 m from the trunk) on the eastern side of the medium tree, about 0.60-0.70 m from the emitters and 0.20, 0.40, 0.60, 0.80 and 1.0 m depth by using a time domain reflectometer (TDR) (Fig. 1).

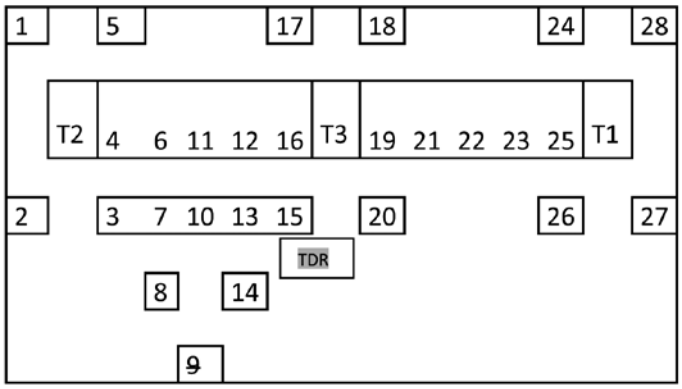

Fig. 1 - Distribution of access tubes for neutron probe measurements taking into account root distribution, soil humectation and soil coverage. Tubes were set around three olive trees of cultivar Chétoui at 1.5 m depth at distances of 1.4 m, 2.2 m, 2.8 m and 4.2 m from trunks.

The heterogeneous distribution of roots and discontinuity of the soil coverage make the interpretation of our measurements difficult. For this reason, four soil compartments designated G_1, G_2, G_3 and G_4 were considered according to the distance to trunk. The groups G_1 and G_2 include measurements of soil water content made below the canopy at 1.4 m and 2.2 m, respectively, with three and two replications. Observations made along the line of drippers at 2.8 m and out of the projected canopy area at 4.2 m belong to groups G_3 and G_4, respectively.

Probe calibration. Calibration of the neutron probe consists in relaying the count ratio (N/N_{water}) and the soil water content values determined for all depths exceeding 0.20 m by concurrently monitoring the probe countings and the gravimetric soil moisture. Measurements were made at the same sites, weekly, in dry and humid conditions to cover all potential values, and more frequently during the irrigation period. Before and after sampling, the counting was sampled in a water medium in order to control the possible drift in the electronic device provided by the probe itself. The average value was used to adjust the measurements made on the same day. Also, we have considered for each trench of soil a specific value of the bulk density (d_a) instead of using an average value for all soil layers, which may increase the error intervals. Finally, the series of data were correlated considering each trench of soil separately. The correlative equations were used to translate the counting values obtained during all the campaign into H_v estimates.

Soil water storage. Water stored in the soil was determined for each trench of soil and then for the whole profile to a depth of 1.2 m using the following equation: S (mm) = 10 x H_v x D, where D = is the layer depth (0.20 m), assuming a standard error of 0.02 m^3m^{-3} on H_v measurements.

Analyses of results. Soil water content values determined for each compartment (groups G_1, G_2, G_3 and

G_4) were analyzed separately considering two temporal scales. The first analysis was made during the campaign and the second concerned some typical days representing the main physiological processes that evolved during the growing season. The first date (29/5/2003), designated (S), coincided with the rapid fruit growth stage and it was dry, without any rain or irrigation supplies. The second date (16/7/2003) was chosen during an irrigation episode, corresponding to the stage of flower induction, designated (I). The third date (23/09/2003) was the period of fruit enlargement, designated (P). It corresponds to a high soil moisture period and was chosen after the first heavy rains (90 mm) of that autumn. Through measurements taken on these dates, we analyzed soil behavior under well- and low-watered conditions and different climatic demand.

3. Results

Calibration of the neutron probe

Count ratios (N/N_{water}) and soil water content values (H_v) obtained gravimetrically were positively correlated with r correlative coefficients (r) ranging between 0.69 and 0.83 for the portion of soil from 0.20 to 1.20 m depth (Fig. 2). The curves established for the four medium layers (20-40 cm, 40-60 cm, 60-80 cm, 80-100 cm), drawn with either the same or different values of bulk density, provided the same coefficient of correlation (r= 0.83, r= 0.76, r= 0.70 and r= 0.73 for 0.20-0.40 m, 0.40-0.60 m, 0.60-0.80 m, 0.80-1.00 m, respectively) for each soil layer. The trench of soil from 1.00 to 1.20 m showed some deviation with r= 0.69 when using a specific da value, and r= 0.66 when using an average value of da. These differences are due to transition between the clay-loamy and clay-sandy soil layers.

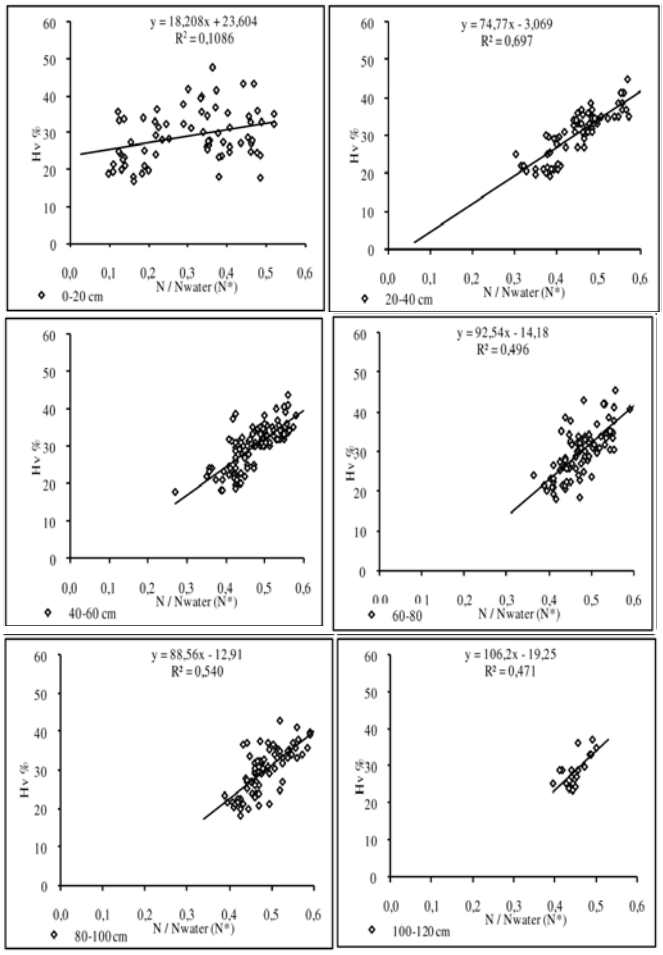

Fig. 2 - Probe calibration curves determined for the neutron SOLO 25 for different soil layers. Gravimetric measurements (H_v) were correlated to the counting ratio $N^*= N/N_{water}$, where N and N_{water} referred to counting made into the soil and water, respectively.

Relationships between TDR, neutron probe and gravimetric soil water content measurements

Measurements of H_v values taken using aluminum and PVC-polyamide access tubes were inter correlated, showing a positive and significant correlation curve with r = 0.73 (Fig. 3). The TDR-measurements were also correlated to the neutron probe estimates and to the gravimetric observations with high correlative coefficients of 0.87 and 0.79, respectively (Fig. 4). These results are of practical interest as they allow use of any apparatus or method with confidence depending on their availability. However, it is important to take into account the representativety of the measurements which could not be taken simultaneously every time at each location (the case of TDR).

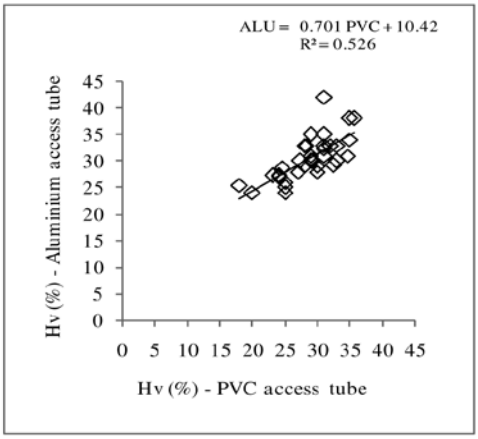

Fig. 3 - Relationship between H_v (%) measurements made simultanuously with the aluminium and PVC-polyamide access tubes.

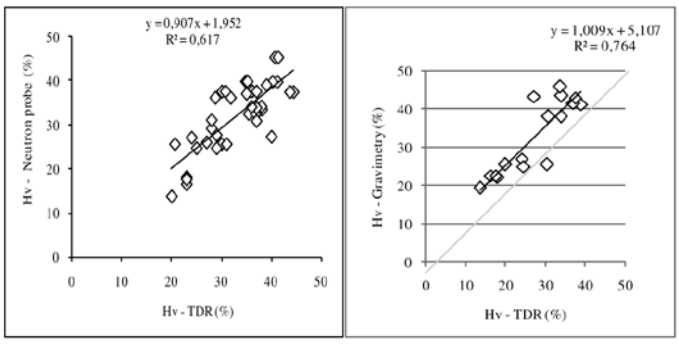

Fig. 4 - Relationships between H_v (%) measurements made simultanuously with TDR, neutron probe and gravimetry.

Spatio-temporal variability of soil water content

Soil water contents measured with TDR fluctuated during the growing season by 15 to 46% depending on depth, season and watering conditions (Fig. 5). During the irrigation period (beginning from 15 May), H_v-values ranged from 25 to 39% with maximum and minimum values observed within the medium depths and at the top soil layer, respectively. Resumption of irrigation at the end of August provided a significant increase of H_v values which decreased rapidly after mid October. The lowest values were recorded at the end of the year under low soil evaporation conditions and root activity, and specially in the upper layer.

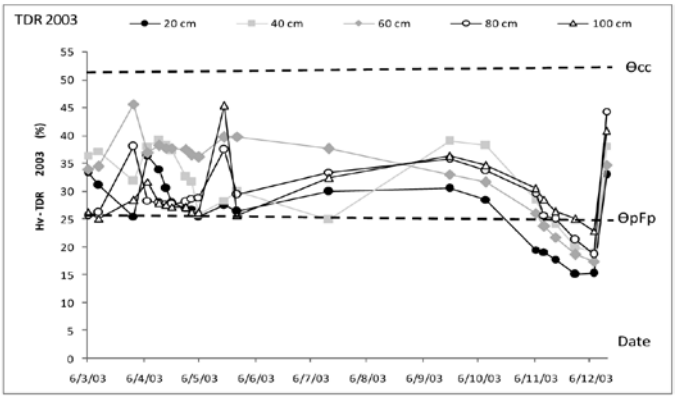

Fig. 5 - Evolution of the TDR-soil water content measurements according to depth in 2003.

Soil water content also varied according to the distance to trunk (Fig. 6). This was observed through estimates of water stocks determined throughout the campaign for all soil compartments (G_1, G_2, G_3 and G_4). Considerable variability was observed between these reservoirs with regard to soil humectation events (i.e. rainfall and irrigation supplies) with values between 225 and 400 mm.

The stock of water recorded at the beginning of the campaign (April) was close to 350 mm with little variability between soil compartments. It then decreased during the first decade of May in response to the increasing climatic demand and plant activity. The beginning of irrigation on 15 May marked the start of a consistent increase of the stocks around the emitters, enhancing the disparities between compartments. Values subsequently evolved continuously with a downward trend following water application. From mid July to the end of August, irrigation was interrupted, leading to a significant decline of these stocks, to reach their lowest value of 225 mm in G_4. Rainfall received in the autumn increased and homogenized the soil water status. The highest stocks were recorded in this case for G_1, while reservoirs G_3 and G_2 provided intermediate values; these areas were not subject to irrigation but they were partially shaded during the diurnal period (reduction of soil evaporation). Reservoir G4 showed the lowest stocks in summer, but it gave the highest values during the rainy period. These results indicate that soil water status mainly depends on depth, water application and distance to trunk, but it may vary depending on plant activity.

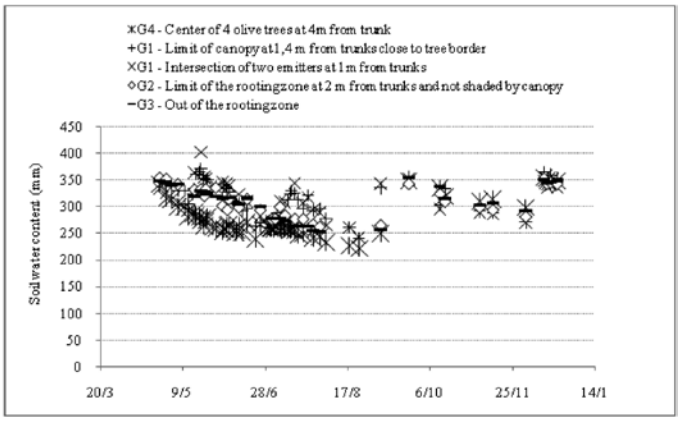

Fig. 6 - Soil water stocks (mm) determined for all the profile (0-100 cm depth) at 1.4 m (G_1), 2.0 m (G_2), 2.8 m (G_3) and 4.2 m (G_4) from trunks.

Soil water content and tree response

Root growth and canopy relationship. Roots extended rapidly during the growing season to reach in May the limit of the canopy at 2.12 m from trunks. Maximum root number was observed at 0.40-0.60 m depth (Table 4), i.e. at depths characterized by high soil water contents (Fig. 5), while maximum root densities (dr) were recorded in the top soil layers at 0.40 m from the trunks (Fig. 7). The highest value of dr (0.67 cm/cm^3) was observed in G_1. Then, as distance to trunk increased, root densities decreased, as did the stocks of water. At a greater distance from trunks (80 cm), maximum root densities ranged between 0.15 cm/cm^3 (deeper layers) and 0.35 cm/cm^3 (upper layers). During this same period, the canopy diameter increased at similar rates leading to equilibrium between the above- and underground areas, just a few years after planting. We recorded at the end of the campaign an optimum LA/L_r (leaf area/root length) value of 2.3 km/m^2 while the ratio S_r/S_c (root area/projected canopy area) approximated the unit (Table 5). This result indicates that as leaf area increased, the amount of carbohydrates increased allow-

Table 4 - Root distribution, number and diameter observed during the experimental year for six-year-old Chétoui olive trees compared with measurements made on five-year-old tree

Soil layer (cm)/Age	5-year-old tree	6-year-old tree
Distribution of roots		
0-20	9	51
20-40	5	91
40-60	3	116
60-80	5	97
80-100	3	81
100-120	0	36
Total number of roots	25	472
Maximum root diameter (mm)	24	27
Volume of the rooting system (cm^3)	5.3	11.2
Maximum distance to trunk (cm)	150	212

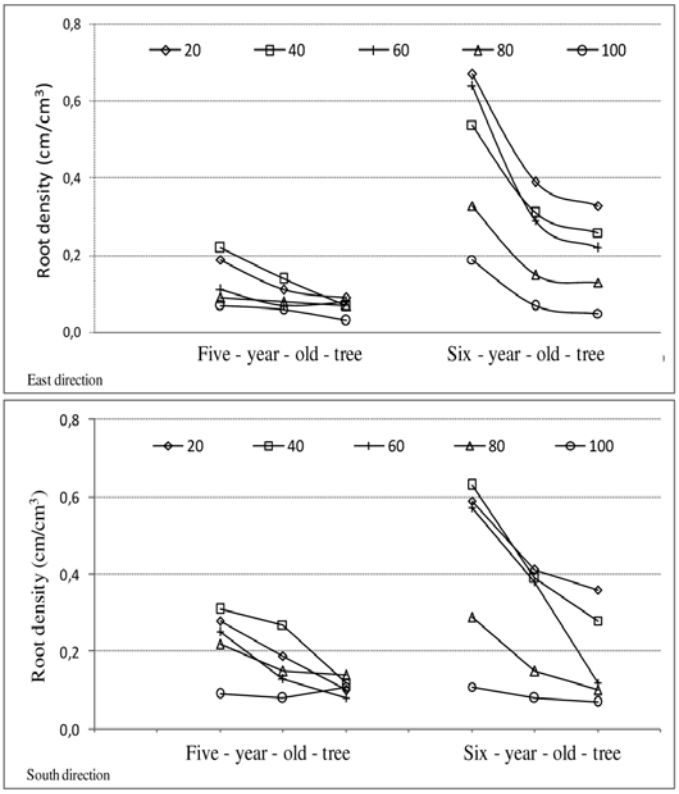

Fig. 7 - Average root densities (cm/cm^3) recorded for five- and six-year-old olive trees at 0.40, 0.80, and 1.20 m distance from trunks. Measurements were made in both east and south directions at different depths (0-20 cm, 20-40 cm, 40-60 cm, 60-80 cm and 80-100 cm).

Table 5 - Characteristics of the rooting system of the six-year-old-tree of cultivar Chétoui compared with those recorded for a five-year-old tree

Soil layer (cm)/Age	5-year-old tree	6-year-old tree
Root area (m^2)	7.10	13.8
Projected canopy area (m^2)	8.04	11.94
Root area (S_r, m^2) / Projected canopy area (S_c, m^2)	0.9	1.2
Maximum canopy radius (m)	1.60	1.95
Average root density (cm/cm^3)	0.13	0.30
Length of the rooting system (km)	7.05	33.94

ing good development of roots and fruits. This hypothesis is analyzed in the following section through simultaneous monitoring of fruit growth and tree height.

Watering conditions, tree growth and fruit development. Growth patterns relative to tree height and fruit diameter observed in 2003 were different from those recorded for the previous year (Fig. 8). In 2002, tree height and fruit diameter increased with irregular rates, but continuously from April till October, peaking at 1.4 cm/day (105 DOY) and 0.22 mm/day (142 DOY), respectively. In the following year, we did not record any peak values for tree height but rather a low and constant rate of about 0.10- 0.15 cm/day. From April to June, fruits grew with increasing rates to peak at 0.29 mm/day on 155 DOY. Soil water contents recorded during this period of cell division and early fruit growth (10/4-10/6, 100-160 DOY) (Fig. 9) were apparently sufficient to assure suitable fruit development.

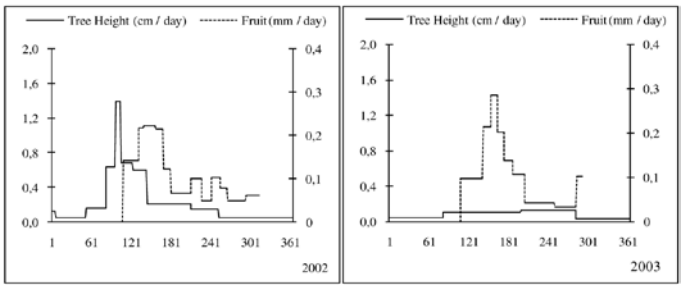

Fig. 8 - Growth patterns of olive tree height (cm/day) and fruit diameter (mm/day) recorded in 2003 compared to tree height and fruit development curves recorded in 2002. Values are averages of 96 tree height and 480 fruit diameter measurements made on Chétoui olive trees.

Tree height and fruit growth decreased significantly in July-August most likely because of interruption of irrigation and the decrease of soil water content values (Fig. 9). After this period of a lack of water, Hv-values increased particularly in the deeper depths, enhancing the ultimate fruit development (284 DOY, 0.1 mm/day).

Soil profiles established in May, July and September showed different behavior depending on the watering conditions and the climatic demand. For measurements made at 1.4 m from trunk (G_1), soil water content values ranged

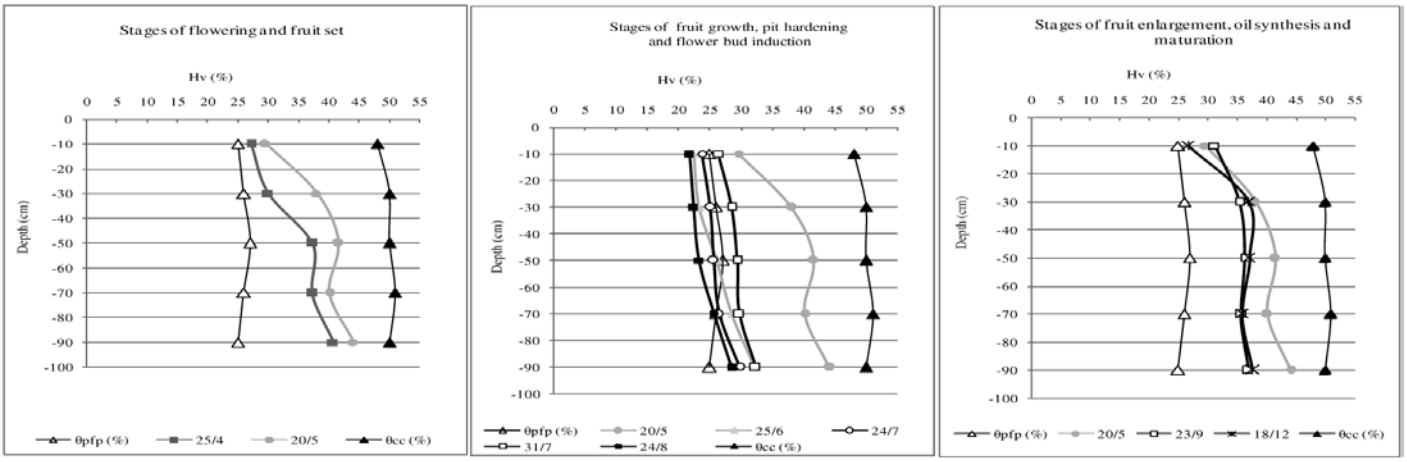

Fig. 9 - Soil water content curves observed at different stages of fruit development.

between 30 and 42% (Fig. 10), providing distinguishable profiles with constant differences between the lowest and the highest values within each trench of soil. However, minimum and maximum soil water contents were not observed during the driest (S) and the wettest period (P), respectively. Minimum values of H_v were recorded in the first 40 cm in May (S) and at deeper depths (0.40-1.20 m) in July (I), while maximum values were recorded in July in the superficial top layer, in September for the 0.20-0.60 m layer and in May at deeper depths (0.60-1.20 m).

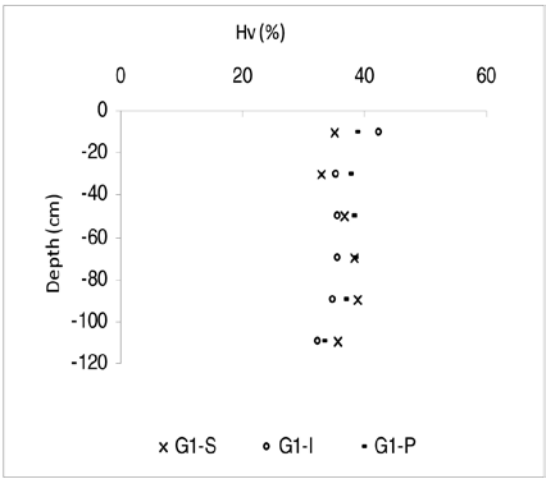

Fig. 10 - Soil water content (H_v, %) measured within the first reservoir (G_1). Measurements were made to 1.2 m depth at a distance of 1.4 m from trunks on May (29-05-2003) after a 20-day period of dryness (G_1-S), in July (16-07-2003) during an irrigation period (G_1- I) and in September (23-09-03) following the first heavy autumn rains (G1-P).

Groups G_2, G_3 and G_4 showed important variations between H_v values within the top soil layers (0-0.40 m) and small differences at deeper depths despite their distances from trunks (2.0 m, 2.8 m and 4.2 m) (Fig. 11). Minimum values (20%) were recorded in May and July despite the different climatic conditions, while the highest values of soil water content were about 40 % at all depths.

4. Discussion and Conclusions

This case study demonstrates the potential of using the neutron probe, TDR and gravimetric measurements to determine soil water content in a young olive orchard, taking into account the heterogeneous distribution of roots, localized irrigation and low canopy shade. Values of H_v were obtained by using the probe calibration curves established specifically for the SOLO 25 apparatus for each trench of soil. These curves cannot be used in any other situation.

Data collected with the different methods and apparatus showed positive correlations between soil water content measurements. This result is of practical interest because it indicates that any of these apparatus or methods can be used depending only on their availability. For example, the relationship developed between the two types of access tubes allowed us to use with confidence the PVC-polyamide tubes which were assembled locally with a lower cost. Regarding the apparatus, the TDR with probes installed vertically has proven to be fast, accurate and non-destructive, but it allowed measurements in one location only. This is a major disadvantage. On the contrary, gravimetric measurements can be made at any location and the method is suitable for calibrating other methods, although it is destructive and hard to carry out. Measurements made with the neutron probe are difficult. The apparatus requires specific calibration and some care should be taken when using such radioactive probes. With regard to these 'constraints', uncertainties arise because soil water content monitoring is incomplete as it is impossible to do measurements at all depths and in all directions. For the neutron probe, it is important to know if the observed variance of water content measurements is really due to errors associated with the location, which can be randomly distributed or spatially structured, or to errors arising from the use of a neutron probe itself. Indeed, uncertainty intervals are also influenced by the charge of the probe battery, the number of access tubes used, their placement and the depths at which they were installed. The number of access tubes should be determined depending on the heterogeneity of the or-

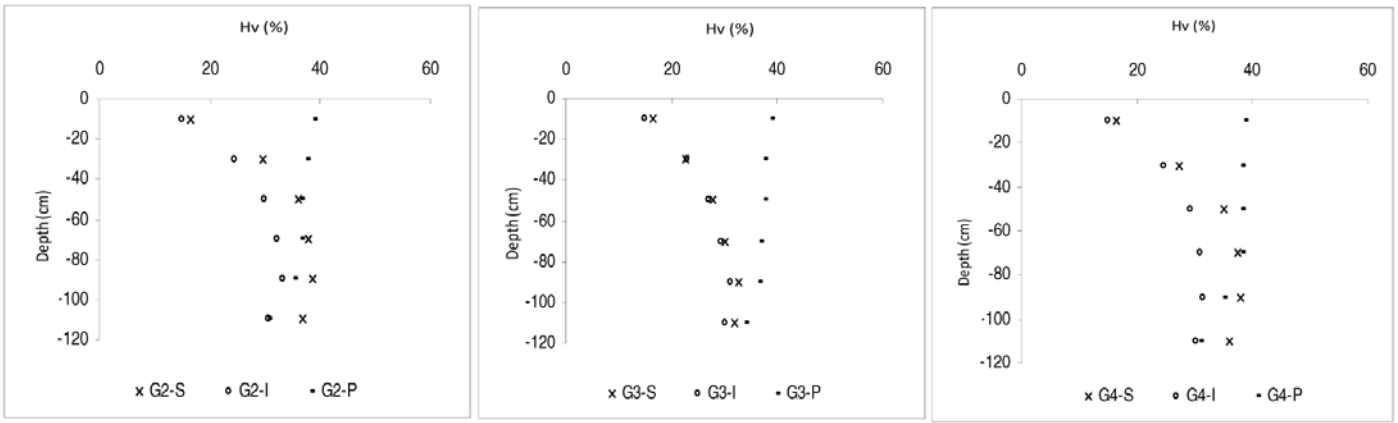

Fig. 11 - Soil water content (%) measured within the second (G_2), third (G_3) and fourth (G_3) reservoirs. Measurements were made to 1.2 m depth at distances of 2.0, 2.8 and 4.2 m from trunks, respectively, in May (29-05-2003) after a 20-day-long period of dryness (G_1-S), in July (16-07-2003) during an irrigation period (G_1-I) and in September (23-09-2003) following the first heavy autumn rains (G_1-P).

chard in order to avoid situations where sample data size is not sufficiently distributed in the field. About 25 locations would be necessary to obtain a mean value with a relatively high precision; observations are thus considered independent of one another regardless of their location in the field. It is also obvious that when installing soil-water measuring devices in the plant row in the irrigated field, at least one device should be located in each of the major soil types to take into account all soil occupation cases. Additional care should be taken with regard to possible drift in the electronic device: (1) counting in a water medium before and after profiles are sampled, (2) using the apparatus with a fully charged battery and (3) the use of a specific d_a value for each trench of soil rather than an average value for all soil layers when the soil texture is variable. However in light of recent concepts introduced in soil physics studies, and as previously stated, it is obvious that the auto-correlation between measurements in estimating the variance of the mean must be taken into account.

Soil water contents varied consistently according to the proximity of measurements to trunk and depth. Maximum variations were observed in the first top soil layers at 0.40 m from trunks as a result of the heterogeneous distribution of roots. There was a massive presence of roots near the trunk, mainly confined to the canopy projected area (Masmoudi *et al*., 2007; Masmoudi-Charfi *et al*., 2011). These results are concordant with those reported by Bonachela *et al*., (1999), Fernandez and Moreno (1999), Palese *et al*., (2000), Fernandez *et al*., (2003) and Connor and Fereres (2005) regarding high root densities in these areas. Some roots were also found at greater distances from the trunk, outside the canopies, i.e. within the reservoirs G_2 and G_3 but with lower densities in comparison to values obtained within the first compartment. This indicates that root uptakes are still possible in these areas, and these roots may have a significant role in enhancing root absorption and water transfer. Furthermore, results showed that the zones where roots develop behave differently, even below the canopy, involving different processes of water uptake and depletion. Unfortunately it was not possible to separate these processes in the present study to determine if these roots are more active than those located near the trunk or not. Fernandez and Moreno (1999) and Connor and Fereres (2005) explain that root densities are necessarily higher in the area of irrigation but roots may be less active. On the contrary, roots far from the trunk may be larger with numerous fine roots and thus they are more active. In another study carried out during the same year, Abid-Karray (2006) reported the presence of lateral water transfers within an olive orchard cultivated in central Tunisia under complementary irrigation. High water depletion was observed in that olive orchard and others cultivated under semi-arid and arid climates due to advective transfers of heat in soil (Fernandez *et al*., 1990; Fernandez *et al*., 1991; Villagra *et al*., 1995; Bonachela *et al*., 1999; Granier *et al*., 2000; Fernandez *et al*., 2003). This makes the situation more complex because roots situated outside of the projected canopy limit may contribute significantly to supply other reservoirs. Their role is however, highly dependent on the distance from the point of water but also the stage of development. We have published in previous papers (Masmoudi-Charfi and Ben Mechlia, 2007 and 2008) that under irrigated conditions young olive trees cultivated in this same location continued to grow even during the winter months, under different watering conditions (extreme rainy and rainless years), however with relatively low rates. Variability of soil water content is also dependent on soil coverage, which varied consistently from year to year, following the season, the severity of pruning and measurement site, thus modifying significantly the relative importance of the evaporative processes involved within each soil reservoir (Dichio *et al*., 2002; Masmoudi *et al*., 2004; 2007). This is because the contribution of the different processes of water uptake and water depletion depend on the amount of solar radiation intercepted by the tree canopy, which is the most important factor controlling water losses and extension of the leaf area. Water applied during the growing season seemed to be sufficient to meet the overall tree water needs although some water shortage was observed during the fruit set-maturation period. Stocks of water recorded at the beginning of the growing season (in May) were apparently insufficient to insure suitable growth of the tree, but high enough to assure early fruit growth. However, the lack of water observed from mid- July to end of August under high evaporative demand, reduced both tree height and fruit size (-10%), fruit weight (-26%) and also fruit number through an important fruit drop observed early September. Comparative results between the year of study and the preceding year showed a significant reduction of yield at harvest. Six-year-old olive trees yielded 1.9 T/ha (2003 was normally an 'on' year), while yield exceeded 2.0 T/ha in 2002 ('off' year). These different responses may be inheritent to other exogenous factors like soil type, pruning, and fertilizer schedules (Masmoudi-Charfi and Ben Mechlia, 2009) or to some endogenous parameiters which have an impact on how irrigation changes affect the production and growth levels. In September, although water was abundant and trees were loaded with fruits, water uptakes seem to be reduced due to the decrease of climatic demand. These results are supported by observations of sap fluxes recorded during the same period (Masmoudi-Charfi *et al*., 2011; 2013), showing that when water is available, sap flux measurements increased in correlation with the increasing climatic demand.

This experiment, concerning the choice of devices used to measure soil water content, despite the observed constraints, has practical interest. The positive correlations developed between soil water content measurements made with the different methods and apparatus indicate that any of these devices can be used indifferently, depending only on their availability and ease. Particularly, the relationship developed between the two types of access tubes allowed us to use with confidence the PVC-polyamide tubes assembled locally with a lower cost. This study has shown also that soil water content consistently affects tree height

and fruit growth rates and varies depending on the depth and distance from the trunk. This spatio-temporal variability makes it difficult to provide proper assessment of the components of the water balance, if it is used for water consumption estimation in young orchards. Therefore, associating methods should be used that make it possible to distinguish between soil evaporation and water lost by transpiration like sap flux measurements. Additional measurements of root activity are also necessary to confine intervals in simulated uptake distributions. Nevertheless, these methods and estimations remain useful tools to decide when and how to irrigate, how much water is stored in the soil for plant use (soil water logging capacity) and to determine allowable water depletion.

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The sustainability of old grapevine mother plants in relation to new mandatory diagnostic tests for virus control

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Key words: Arabis mosaic virus, Grapevine fanleaf virus, Grapevine leafroll, Grapevine virus A, phytosanitary test.

Abstract: In 2011, the methods to perform phytosanitary tests to check for viruses in grapevine nurseries were reassessed and new regulations were defined (DM 13 December 2011). The mandatory tests require serological assays for the diagnosis of five viruses in grapevine mother plants transplanted in Tuscany in 2001 or before. The aim of the present paper is to report the impact of certification programs applied before 2001 in Tuscany and the sustainability of older mother plants with relation to the new mandatory diagnostic tests. Among the cultivars, virus infection was reported in 19.2% of pool samples, whereas 2.4% of rootstock pool samples showed a compromised health status. GLRaV-3 is the most frequently found virus (10.4% and 1.3% of cultivar and rootstock pools, respectively), and it is also included in the most frequent multiple infections. Multiple infections represent about 25% of infected cultivar pools and almost 50% of infected rootstock pools.

1. Introduction

Italian production of propagated certified grapevines by nurseries is regulated by laws (DM 8 February 2005; DM 7 July 2006) that define the procedures to obtain a certification for propagative material in order to handle healthy plants. These regulations provide detailed guidance for the registration of the primary source to be maintained by the conservative breeder and for the production of basic material or mother plants, the latter grown in nurseries and used to produce certified materials delivered to the growers. In 2009, the "Working group ARNADIA - grapevine viruses" was established, within the Italian Ministry of Agriculture Finalized Project "ARNADIA", with the purpose of producing validated reference diagnostic protocols for the control and monitoring of plant pathogens of phytosanitary interest. In 2011, the methods proposed to perform phytosanitary testing were reassessed (DM 13 December 2011) and it was established that mother plants grown in Italian nurseries (cultivars or rootstocks) have to be checked for virus infections 10 years after transplanting. The first deadline was set for 30 June 2012 to consider mother plants transplanted in 2001 or earlier. Obviously, these mother plants were checked and produced according to older regulations (starting with DPR 24 December 1969) that define pathogens (absence of grapevine leafroll and fanleaf degeneration disease) and methods for their assay (biological indexing) differently compared to the most recent regulations. Presently, plants are tested for the following viruses: Grapevine leafroll associated virus -1 (GLRaV-1) and -3 (GLRaV-3), Grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV) and Grapevine virus A (GVA). The significant presence of these viruses was recently reported in Tuscany after assays carried out in sanitary selection programs. From 1997 to 2004, health tests conducted on 172 uncertified plants selected from the Tuscan costal area (Elba Island, Lucca and Maremma) showed a critical phytovirologic condition, with 97.1% infected plants (Materazzi *et al*., 2006 a). In the period 2000-2004, health tests conducted on 318 uncertified grapevine plants selected in D.O.C. or D.O.C.G. areas of Tuscany (Chianti Classico, Montalcino and Montepulciano) revealed that 58.8% vines were virus-infected (Materazzi *et al*., 2006 b). These findings cannot be transferred to nurseries, considering their use of certified materials. In any case, the virologic status of uncertified grapevine in Tuscany indicates the presence of grapevine viruses and related vectors, underlining the importance of periodic verifications in grapevine nurseries to guarantee the highest health standards of plant production and to help reduce

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the spread of grapevine pathogens, as determined by the updated regulations.

In this paper, we report the results obtained from serological (ELISA) tests for the diagnosis of five viruses in grapevine mother plants (cultivars or rootstock) transplanted in 2001 or before, as dictated by DM 13 December 2011. The aim is to report the impact of certification programs applied before 2001 in Tuscany and the sustainability of older mother plants as stated by the new mandatory diagnostic tests.

2. Materials and Methods

Plant sampling and ELISA tests

Plant sampling and ELISA tests were carried out following procedures defined by the Working group ARNA-DIA - grapevine viruses, included in DM 13 December 2011. In accordance with legislation, the following steps were undertaken. Sampling was performed beginning in November 2011 in 33 nurseries, collecting sample pools composed by homogenous material from five plants. Phloem tissue (2 g) was collected from pools and mechanically ground (Tissue Lyzer with 10 ml-grinding jar, Qiagen, Venlo, Netherlands) with extraction buffer. ELISA test was performed using commercial polyclonal antibodies as well as negative and positive controls (Agritest, Bari, Italy). Absorbance at OD_{405} nm was recorded by photometry (Titertek multiskan, Titertek Instruments Inc., Huntsville, USA). Readings were normalized as R value (OD-treated explant/OD-HC), identifying the R= 2 threshold which distinguishes the positive versus the negative response (Monette, 1983).

3. Results

The ELISA test (Table 1) showed that 19.2% of cultivar pools were infected with at least one of the viruses. All five viruses were found in cultivar samples, but GLRaV-3 was considerably more frequent than the others, followed by GVA. The combination of these two viruses also represent the most frequent multiple infection. Multiple infections represent about 25% of infected pools and they are characterized by 13 different virus combinations. The least frequent virus was ArMV.

With regard to rootstock pools, there was a low rate of infection (2.3%), even if GLRaV-3 was still the most frequent virus detected. This virus was also included in the most frequent multiple infections that, for rootstocks, represent almost 50% of infected pools. Multiple infections were reported in ten different virus combinations. Also in rootstocks, ArMV was the least frequent virus.

4. Discussion and Conclusions

Monitoring revealed GLRaV-3 as the most frequent virus in cultivar or rootstock mother plants, as reported in sanitary selection research previously carried out in Tuscany (Triolo and Materazzi, 2004; Materazzi *et al*., 2006 a) and other Italian areas (Digiaro *et al*., 2000; Bica *et al*., 2002; Martelli, 2002). Similarly, the low frequency of ArMV is in agreement with other health checks performed in Tuscany on uncertified plants (Borgo *et al*., 2000; Materazzi *et al*., 2006 a). Viruses were frequently detected in multiple infections, in particular in rootstocks, with a wide range of combinations.

Table 1 - Rates of virus infection for cultivar or rootstock pools detected by ELISA test

Mother plant	No of checked pool		No of infected pool	% of virus infection	
Cultivar	712		137	19.2	
Rootstock	1523		36	2.4	
Infected pool out of total (%)	GLRaV-1	GLRaV-3	GFLV	ArMV	GVA
Cultivar	2.2	10.4	1.8	1.4	5.1
Rootstock	0.9	1.3	0.9	0.6	0.7
	Pool infected by multiple viruses out of total (%)				
	Cultivar			Rootstock	
GLRaV-3/GVA	2.11		GLRaV-1/GLRaV-3	0.33	
GLRaV-1/GLRaV-3/GVA	0.56		GLRaV-3/GVA	0.13	
GLRaV-1/GLRaV-3/GVA/GFLV	0.42		GLRaV-3/GFLV	0.13	
GLRaV-1/GLRaV-3	0.28		ArMV/GVA	0.13	
GLRaV-1/GFLV	0.28		Others	0.46	
GLRaV-3/GVA/ArMV	0.28				
Others	0.98				
Total multiple infections	4.91		Total multiple infections	1.18	

Considering that these findings represent the first application of DM 13 December 2011, it is not possible to evaluate the health status of mother plants grown in Tuscan nurseries. Moreover, comparison to other Italian areas is not relevant because no homogeneous data are available. In any case, these finding can be a starting point to evaluate the health trend of plants in Tuscan nurseries.

The current health status of mother plants may be due to re-infection events as all tested viruses are known to be vector-transmitted (Golino *et al*., 2002; Andret-Link *et al*., 2005; Zorloni *et al*., 2006; Demangeat *et al*., 2010; Tsai *et al*., 2010) and relative vectors have been found in Tuscany. However, the significant improvement in diagnostic tests over the last 30 years do not seem to exclude that the primary source or basic material were originally infected. Even if these categories are considered in DM 13 December 2011, there is no updated health information available that can reconstruct the propagation links. In this case, the application of traceability tools such as electronic identification (Bandinelli *et al*., 2009; Luvisi *et al*., 2012 a, b) could support retrieval of health information. Moreover, the activity of local conservative breeders, such as the Associazione Toscana Costitutori Viticoli (TOS.CO.VIT.) set up in Tuscany in 2003 (Triolo, 2011), can promote the use of certified plants selected according to the most recent regulations.

Considering that the rate of infected cultivars and rootstocks was found to be very low during verifications carried out during Tuscan sanitary selections, these findings confirm that the use of certified plants helps reduce the spread of grapevine viruses.

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Effects of chicken manure and vermicompost teas on herb yield, secondary metabolites and antioxidant activity of lemon basil (*Ocimum* × *citriodorum* Vis.)

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Key words: essential oil, flavonoids, organic agriculture, sustainable agriculture, total phenolics.

Abstract: Effects of chicken manure tea (CMT) and vermicompost tea (VCT) as soil drench on vegetative growth, herb yield, essential oil content, total phenolics, total flavonoids and antioxidant activity of lemon basil (*Ocimum* × *citriodorum* Vis.) was evaluated in a two-year field experiment. The greatest plant height, number of leaves and flowers, shoot fresh and dry weight and leaf chlorophyll content were obtained using CMT at either 1:5 or 1:10 dilutions with no significant differences. The highest number of lateral branches and flavonoid content were obtained when CMT at 1:5 dilution was applied. Essential oil content was at its highest level (0.618%) when CMT or VCT were used at 1:10 dilution, while the greatest total phenolic content and total antioxidant activity were obtained at 1:5 dilution of VCT. The results emphasize the possibility of using organic-based compost teas for enhancing herbal yield and important secondary metabolites in aromatic medicinal plants.

1. Introduction

The genus *Ocimum* (*Lamiaceae* family), collectively called basil, comprises between 50 and 150 species of herbs and shrubs (Darrah, 1980). Basil is native to Asia (India, Pakistan, Iran, Thailand, and other countries) and can be observed growing wild in tropical and sub-tropical regions (Makri and Kintzios, 2008). The essential oil profiles of this group of plants are extremely variable, such that several aroma compounds can be found in chemotypes of basil such as citral, eugenol, linalool, methylchavicol, and methylcinnamate that are traded in the international essential oil market (Simon *et al.*, 1999). The diversity within basil species has been accentuated by centuries of cultivation and cross compatibility, which has lead to great variation in morphology and chemical profile (Javanmardi *et al.*, 2002). Basil species have antioxidant, antimicrobial and antitumor activities that are due to the presence of phenolic acids and aromatic compounds (Hussain *et al.*, 2008).

Lemon basil (*Ocimum* × *citriodorum* Vis.), a hybrid of sweet basil (*Ocimum basilicum*) and American basil (*Ocimum americanum*), is a herb grown primarily in northeastern Africa and southern Asia (Fisher and Phillips, 2006). It is naturalized in Asia and cultivated for its lemon-scented leaves due to the essential oils citral and neral as predominant compounds (Grayer *et al.*, 1996). Lemon basil is characterized by its small stature, early flowering, and small, narrow leaves.

Application of organic sources of nutrients, with no or very little use of inorganic fertilizers, is rapidly gaining favor (Anwar *et al.*, 2005). Compost tea is a highly concentrated microbial solution produced by extracting beneficial microbes from compost. Compost tea is produced by mixing compost with water and incubating it for a defined period, either actively aerating (aerated compost tea, ACT) or not (non-aerated compost tea, NCT) and with or without additives that are intended to increase microbial population densities during production (Scheuerell and Mahaffee, 2002; Ingham, 2005). It is a source of foliar and soil nutrients, contains chelated micronutrients for easy plant absorption and the nutrients are in biologically available forms for both plant and microbial uptake (Hendawy, 2008). Many researchers have pointed out the efficacy of organic manures, compost and compost teas in increasing vegetative growth, biomass and essential oil yield of sweet marjoram (Gharib *et al.*, 2008), cumin (Safwat and Badran, 2002), fennel (Azzaz *et al.*, 2009) and sweet basil (Khalid *et al.*, 2006). Improvements in yield and quality following application of these organic-based substances has been attributed to an enhancement of the beneficial microbial communities in soil, an improvement of mineral absorption conditions for plants, and a stimulation of defense compounds, growth regulators or phytohormones in

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plants (Pant *et al.*, 2009). Various liquid manures or their extracts are known to serve primarily as a source of soluble plant nutrients, growth stimulants and disease suppressors (Khalid *et al.*, 2006).

Several studies have reported the effects of compost tea on suppression of certain plant diseases such as damping–off caused by *Phytium ultimum* (Scheuerell and Mahaffee, 2004), gray mold (*Botrytis cinerea*) (Scheuerell and Mahaffee, 2006), *Alternaria solani* and *Phytophthora infestans* (Koné *et al.*, 2010). However, relatively little work has been done to investigate the effect of vermicompost or manure teas on yield, nutritional and quality factors, secondary metabolites and antioxidant activity of vegetable herbs.

In the present work the effects of chicken manure and vermicompost teas as soil drench on growth characteristics, chlorophyll content, essential oil yield, total phenolics, total flavonoids and antioxidant activity of lemon basil were evaluated. The significance of this study lies in the possibility of applying compost teas as soil amendments to improve yield components and secondary metabolites in organic agriculture.

2. Materials and Methods

Site description, plant material and experimental design

The experiment was carried out in two subsequent years, 2010 and 2011, in a field 1810 m above sea level in a silty-loam soil. Three soil samples from 0-30 cm depth were collected and sent to a certified local soil laboratory for analysis. The chemical properties of the soil are shown in Table 1. The local maximum-minimum mean temperatures and relative humidity during the growing period were 28.7-12.6°C and 47.3%, respectively.

Seeds of lemon basil (*Ocimum* × *citriodorum* Vis.) were sown in double rows (20 cm apart) with 50 cm spacing. Seedlings were thinned three weeks after sowing to 15 cm between plants within the rows. Plants were harvested at ground level when they were at full bloom stage (90 days after sowing) for further analyses. Irrigation (using a drip-tube system), hand weeding, and other management practices were performed when required throughout the growing period.

The experiment was carried out in a complete randomized block design with three replicates per treatment, each of which consisted of 20 plants. Five fertilization treatments were applied as soil drench of de-ionized water (control), chicken manure tea (CMT) at 1:5 and 1:10 water dilution (v/v) and vermicompost tea (VCT) at 1:5 and 1:10 water dilution (v/v).

Tea preparation and application method

Vermicompost and chicken manure teas were prepared as described by Javanmardi (2010). Briefly, vermicompost and chicken manure were separately mixed with tap water at ratios of 1:5 and 1:10 (v/v) in loosely covered 14 l plastic containers. Water was allowed to stand for 24 h for passive chlorine removal before mixing. The mixtures were aerated using an aquarium pump for 72 h brewing time in a shaded area. Solutions were filtered through cheesecloth before application. Chemical properties for teas are presented in Table 2. Treatments were started four weeks after sowing and applied five times as soil drench with 600 ml of solution per plant at weekly intervals. Fresh solutions were prepared for each application interval.

Vegetative growth parameters

At full bloom stage ten central plants from each replicate (to avoid marginal effect) were cut at ground level and plant height (cm), number of branches per plant, number of flowers, number of leaves, and fresh and dry weight of herb (g per plant) were recorded.

Chemical analysis

Chlorophyll content. Chlorophyll content was determined as described by Saini *et al.* (2001). Randomly selected samples of fully expanded leaves (0.5 g) were used. Samples were homogenized with 5 ml of acetone (80% v/v) using a pestle and mortar and filtered through filter paper (Whatman No. 2). The process was conducted in the dark to avoid photo bleaching. Absorbance was measured with a UV-visible spectrophotometer (Camspec M108, Spectronic Instruments, Leeds, UK) at 652 nm and total chlorophyll content calculated using:

$$\text{Total chlorophyll (mg·g}^{-1}\text{ FW)} = [D_{652} \times V] \times V/W$$

where: V is the total volume of acetone extract (ml) and W, the fresh sample weight (g).

Essential oil content. Quantitative determination of the essential oil obtained from lemon basil subjected to the different treatments was achieved by placing the air-dried herbage in a 2 l flask with distilled water (1:15 w/v) and using a Clevenger apparatus, as described by Charles and

Table 1 - Chemical properties of soil

Organic matter (%)	Total N (%)	Available phosphorus as P (mg·kg^{-1}) Bray method	Available potassium as K (mg·kg^{-1})	Fe (mg·kg^{-1})	Cu (mg·kg^{-1})	Mn (mg·kg^{-1})	Zn (mg·kg^{-1})	pH	EC (ds·m^{-1})
1.21	0.05	13.50	540	4.88	1.19	0.39	0.23	7.73	1.50

Table 2 - Chemical properties of vermicompost tea (VCT) and chicken manure tea (CMT)

	vermicompost tea (1:5)	vermicompost tea (1:10)	chicken manure tea (1:5)	chicken manure tea (1:10)
Organic matter (%)	-	-	-	
Total N (%)	6.30	3.14	2.21	1.10
Phosphorus as P (%)	1.01	0.50	1.31	0.65
Potassium as K (%)	6.18	3.08	5.61	2.81
Fe ($mg \cdot l^{-1}$)	9.20	4.51	9.00	4.48
Cu ($mg \cdot l^{-1}$)	0.07	0.03	5.00	2.48
Zn ($mg \cdot l^{-1}$)	25.0	12.0	3.20	1.60
Mn ($mg \cdot l^{-1}$)	0.18	0.89	0.08	0.04
pH	8.22	8.22	8.29	8.29
EC ($ds \cdot m^{-1}$)	4.41	2.20	2.82	1.41

Simon (1990). The average essential oil content of aerial parts is reported as percent of plant dry matter.

Sample preparation for total phenolic and flavonoid content determination. Samples were prepared using the method described previously by Javanmardi *et al.* (2003). Briefly, 250 mg of dried plant material from each replicate were ground and dissolved in 10 ml of 80% acetone. Sample extracts were rotated for 1 h in the dark and centrifuged at 5400 g for 10 min. One ml of supernatant was dried under vacuum at 45°C and kept at -18°C for further use. Each sample was dissolved in 1 ml acetone prior to analysis for total phenolic and flavonoid determination.

Total phenolic compound analysis. The amount of total phenolics in extracts was determined with the Folin-Ciocalteau reagent using the method of Spanos and Wrolstad (1990), as described by Javanmardi *et al.* (2003). To 50 ml of each sample, 2.5 ml of 1/10 dilution of Folin-Ciocalteau reagent and 2 ml of Na_2CO_3 (7.5%, w/v) were added and incubated at 45°C for 15 min. The absorbance of all samples was measured at 765 nm using a UV-visible spectrophotometer (Camspec M108, Spectronic Instruments, Leeds, UK). Gallic acid was used as standard and results are expressed as mg of gallic acid equivalent per g of dry weight (mg GAE/g dw).

Total flavonoid analysis. The method described by Adom and Liu (2002) was adopted for total flavonoid content analysis. To 0.5 ml of extract, 2.5 ml distilled water were added followed by 0.15 ml of 5% $NaNO_2$ solution. The mixture was left to stand for 6 min at room temperature before adding 0.3 ml 10% $AlCl_3.6H_2O$ solution. The mixture was left for an additional 5 min, then 1 ml of 1 M NaOH added and made up to 5 ml with distilled water. The solution was vortexed and the UV absorbance at 510 nm was recorded against catechin as reference. The result is expressed as µg/g DW.

Total antioxidant activity assay. The antioxidant activity of samples was determined by free radical scavenging activity assay using 1,1-diphenyl-2-picryl-hydrazil (DDPH) reagent according to Brand-Williams *et al.* (1995). The ground leaves (1 g) were extracted with 50% methanol, 50% water. To 0.75 ml of the extract sample, 1.5 ml of freshly prepared methanolic DPPH solution (20 $\mu g \cdot ml^{-1}$) were added and stirred. The decolorizing process was recorded after 5 min of reaction at 517 nm and compared with a blank control. The total antioxidant activity is expressed in % calculated as (control absorbance - sample absorbance /control absorbance) × 100.

Statistical analysis

The experiment was carried out for two years in a randomized complete block design (RCBD) with three replicates, each of which consisted of 10 plants. Data were analyzed using one-way analysis of variance (one-way ANOVA) and means of two years for each trait were compared with Least Significant Difference (LSD) at $p \leq 0.05$ by SPSS12 (SPSS Inc., Chicago, IL) computer software for Windows. The data presented in tables and figures are mean values ± standard errors of two years of data for three replicates.

Pearson correlation analysis using SPSS12 (SPSS Inc., Chicago, IL) was performed to assess the relationship between total phenolics, total flavonoids and essential oils with total antioxidant activity.

3. Results and Discussion

The combined analysis of data from the experiment did not show any significant differences for all traits (data not shown).

Growth parameters

Analyses of variance showed that the growth parameters of lemon basil including plant height, number of lateral branches, leaves and flowers, shoot fresh and dry

weight at full bloom stage were significantly affected by VCT and CMT (Table 3).

Plant height and number of leaves

The greatest plant height and leaf number were observed at 1:5 CMT, which was not significantly different from 1:10 dilution. Other treatments did not show significant differences for plant height and leaf number (Table 3). The same result has been reported in *Plantago* plants under 300 ml·l^{-1} compost tea application (Hendawy, 2008). The increasing effects of organic manure and bio-fertilizers on plant height on fennel (Azzaz *et al.*, 2009) and peppermint (Swaefy *et al.*, 2007) have been previously reported.

Number of lateral branches

The highest lateral branch number was observed at 1:5 CMT dilution, however it was not significantly different from 1:10 CMT. The differences among other treatments (1:5 and 1:10 VMT and control) with regard to plant lateral branches were not significant (Table 3). A promoting effect of organic fertilizers on the number of branches was observed also by Azzaz *et al*. (2009) in fennel plants, especially when organic fertilizers were used in combination with bio-fertilizers.

Number of flowers

The highest flower numbers were observed in lemon basil plants treated with CMT (1:5 and 1:10 dilutions) and VCT (1:5 dilution) with no significant differences. Control plants and VCT-treated plants at 1:10 dilution showed lower flower numbers (Table 3). An increased number of flowers in *Plantago* plants under 300 ml·L^{-1} compost tea application has previously been reported (Hendawy, 2008).

Shoot fresh and dry weight

The highest shoot fresh and dry weights were observed at 1:5 CMT, which was not significantly different from 1:10 dilution. Other treatments did not show significant differences for these parameters (Table 3). The promoting effect of chicken manure tea on herb yield may be attributed to the micronutrient content and to the action of living micro-organisms and microbial metabolites which stimulate plant growth (Diver, 2002; Carpenter, 2005). Higher fresh and dry weights have also been attributed to the availability of macronutrients, especially nitrogen, and/or to the improvement of soil water-holding capacity (El-Sherbeny *et al.*, 2005). Furthermore, it has been stated that organic manure activates many species of living organisms which release phytohormones and may stimulate plant growth and absorption of nutrients (Naguib and Aziz, 2003). The same increased vegetative growth characters (including shoot fresh and dry weights) of basil plants under organic farming has been previously reported (Khalid *et al.*, 2006).

Chemical parameters

Leaf chlorophyll content. The highest leaf chlorophyll content was observed at 1:5 CMT which was not different from 1:10 CMT dilution. Other treatments did not show significant differences for this parameter (Table 3). The promoting effect of highly N-containing chicken manure tea on chlorophyll contents might be attributed to the fact that N is a constituent of chlorophyll molecule. Moreover, nitrogen is the main constituent of all amino acids and lipids that act as structural compounds of the chloroplast (Al-Tarwneh, 2005). In sweet basil, chlorophyll content was significantly higher when organic manure compost was applied than in non-fertilized control plants (Taie *et al.*, 2010).

Essential oil content. The essential oil content of lemon basil was affected by dilution levels of organic compost teas. The 1:10 dilution of VCT and CMT was higher in essential oil content compared to 1:5 dilutions (Fig. 1). The highest essential oil content was found in the 1:10 VCT treatment and it was about 3.12 times higher than in control plants. Previously, the highest essential oil content in *Ocimum basilicum* was obtained following soil application of vermicompost at 10 t·ha^{-1} level compared to application of 10 t·ha^{-1} farmyard manure and control treatments (Anwar *et al.*, 2005). In marjoram plants, aqueous extract of compost increased essential oil percentage and yield (Gharib *et al.*, 2008). In basil as a source of essential oils and aroma compounds (Simon *et al.*, 1990), the increase in essential oil yield has been attributed to increase in vegetative growth or changes in leaf oil gland population (Gharib *et al.*, 2008). Our data are in agreement with a previous work that found a higher essential oil percentage in ba-

Table 3 - Effect of different dilutions of chicken manure tea (CMT) and vermicompost tea (VCT) on plant height, number of leaves, lateral branches and flowers, shoot fresh and dry weight and leaf chlorophyll content of lemon basil plants

Treatment	Plant height (cm)	No. of leaves	No. of lateral branches	No. of flowers	Shoot fresh weight (g)	Shoot dry weight (g)	Chlorophyll (mg·g^{-1} fw)
Control	27.30±0.89	130.03±12.15	6.80±0.84	6.57±0.90	8.10±1.01	1.45±0.18	0.71±0.016
CMT (1:5)	30.47±1.27	225.93±22.80	10.23±0.92	11.80±1.62	17.63±2.44	2.64±0.36	0.82±0.012
CMT (1:10)	29.23±1.09	197.30±22.50	8.07±0.85	10.27±1.48	14.27±2.41	2.51±0.48	0.80±0.013
VMT (1:5)	27.57±0.64	136.70±13.58	6.77±0.93	10.47±1.80	8.37±0.92	1.60±0.18	0.74±0.016
VMT (1:10)	27.30±1.01	131.13±12.88	5.20±0.72	6.84±0.91	7.87±1.07	1.53±0.17	0.74±0.015
LSD value (*p=0.05*)	2.65	47.11	2.34	3.85	4.58	0.79	0.039

Data (per plant) are mean values of 3 replicates ± standard errors.

sil due to application of organic fertilizers as compared to control plants (Taie *et al.*, 2010).

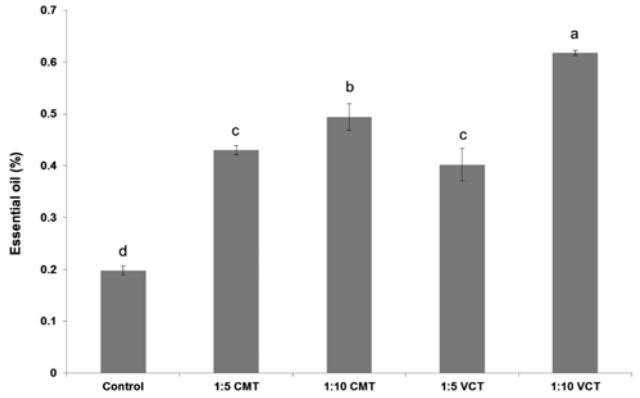

Fig. 1 - Effect of different dilutions of chicken manure tea (CMT) and vermicompost tea (VCT) as soil drench on essential oil percent of lemon basil. Vertical bars show standard errors of the means (n=3).

Total phenolic content. Application of VCT at 1:5 dilution gave the highest total phenolic content. The differences between CMT and 1:10 dilution of VCT were not significant (Fig. 2). Previously, Sousa *et al.* (2005) and Taie *et al.* (2010) reported that total phenolic contents achieved by organic culture were higher than those from conventional practice in tronchuda cabbage (*Brassica oleracea* L. var. costata DC) and sweet basil. Asami *et al.* (2003) and Wang and Lin (2002) also observed consistently higher levels of total phenolics in organically-grown crops compared with those produced by conventional agricultural practices. The amount of total phenolic content of lemon basil in this experiment is in the range of previously reported total phenolic content of different sweet basil accessions (Javanmardi *et al.*, 2003). Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators (Lukmanul-Hakkim *et al.*, 2008).

Total flavonoids. The highest total flavonoid content was observed in 1:5 CMT-treated lemon basil plants and it was over 1.4 times higher than that produced in control plants. The differences between the two dilutions of VCT were not significant (Fig. 3). In previous studies, the amounts of surface flavonoids in *Ocimum x citriodorum* specimens were reported in a range of 0.2 to 5.7 mg g^{-1} (Grayer *et al.*, 2004). Our finding is in agreement with a previous work that reported a significant increase in flavonoid content of *Ocimum basilicum* due to compost or compost tea application in comparison with control plants (Khalid *et al.*, 2006). It has been stated that the antioxidant activity in basil is largely due to the presence of phenolic components, including flavonoids and phenylpropanoids (Juliani and Simon, 2002).

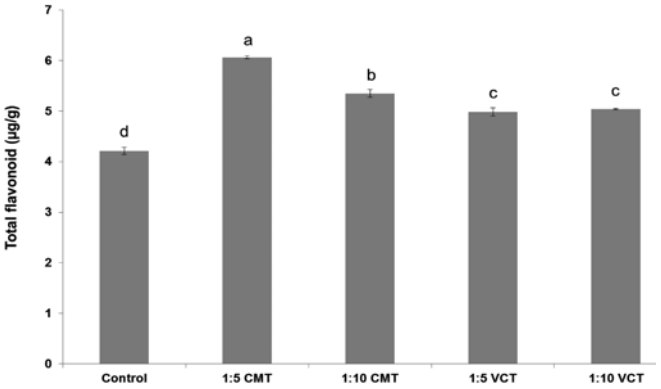

Fig. 3 - Effect of different dilutions of chicken manure tea (CMT) and vermicompost tea (VCT) as soil drench on total flavonoids of lemon basil. Vertical bars show standard errors of the means (n=3).

Total antioxidant activity. The antioxidant activity of lemon basil extracts is shown in figure 4. The total antioxidant activity (TAA) ranged from 48.28% (control) to 58.42% (VCT at 1:5 dilution). There were no statistically significant differences between CMT dilutions and 1:10 dilution of VCT.

Pearson correlation analysis between secondary metabolites (total phenolics, total flavonoids and essential oils)

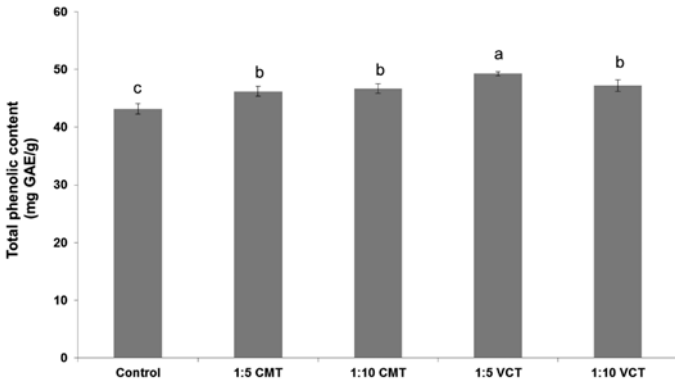

Fig. 2 - Effect of different dilutions of chicken manure tea (CMT) and vermicompost tea (VCT) as soil drench on total phenolic content of lemon basil. Vertical bars show standard errors of the means (n=3).

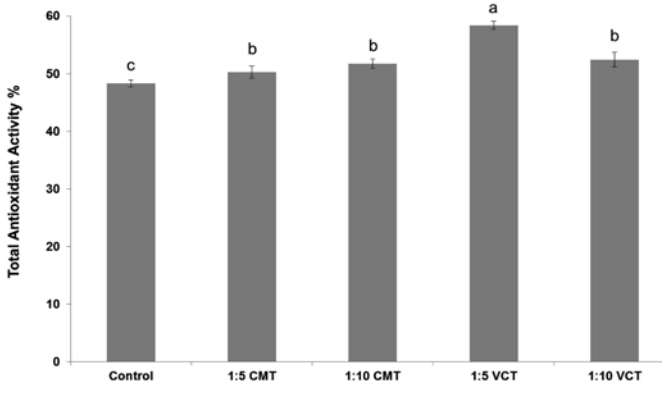

Fig. 4 - Effect of different dilutions of chicken manure tea (CMT) and vermicompost tea (VCT) as soil drench on total antioxidant activity of lemon basil. Vertical bars show standard errors of the means (n=3).

with total antioxidant activity showed a significant positive correlation coefficient of R^2=0.91 between the amount of total phenolics and total antioxidant activity (Table 4). This means that about 91% of total antioxidant activity in lemon basil plants was due to phenolic compounds. Other secondary metabolites did not show significant contributions to the antioxidant activity. The same correlation was previously reported in sweet basil (*Ocimum basilicum* L.) accessions (Javanmardi *et al.*, 2003). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agent, hydrogen donors, single oxygen quenchers and having possible metal chelating activity (Rice-Evans *et al.*, 1995).

Table 4 - Pearson's correlation coefficients between total phenolics, total antioxidant activity, total flavonoids and essential oil percent of treated lemon basil plants

	Total phenolics	Total flavonoids	Essential oil content
Total antioxidant activity	0.919	0.071 NS	0.320 NS

Data (per plant) are mean values of 3 replicates ± standard errors.

4. Conclusions

The findings of this study indicate that fertilization with chicken manure and vermicompost organic teas improves herbal and essential oil yields as well as antioxidative agents such as total phenolics in lemon basil plants. The results point to the beneficial effects of compost tea as possible nutrition sources on growth characteristics and essential oil yields of basil. Organic nutrition systems have environmental advantages such as a beneficial impact on soil properties and the production of safe plants.

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Influence of bagging on fruit quality and mineral composition of Himsagar mango grown in new alluvial zones of West Bengal

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Key words: fruit bagging, fruit quality mineral composition, Himsagar mango.

Abstract: The effect of polybagging of mango (*Mangifera indica* L.) fruits was evaluated at different stages of fruit development (35, 45, 55 and 65 days after fruit set). Fruits were harvested at different stages of maturity (75, 85 and 90 days after fruit set) and allowed to ripen at room temperature (34-36°C, RH 85-90%). The use of bagging at different stages of fruit development improved the appearance of fruit, fruit weight and size through other effects such as increased relative humidity and a consequently reduced fruit water loss. The maturity of fruits, at all stages of fruit harvest, was delayed with increasing bagging duration. Early bagging of fruit (35 days after fruit set) delayed the development of ripening characteristics in comparison to delayed bagging and unbagged control fruit, which ripened earliest. This was clearly evident from the carotene content in the mango flesh, at the different stages of harvest and of ripening fruit, which was the result of higher temperature inside the bags. In bagged fruits usually day/night temperature fluctuations were reduced and there was a cut off in the temperature curve inside the bag. The total soluble solids and sugar content were higher and titratable acid content was always less in unbagged fruit at all stages of fruit harvest and fruit ripening. Mineral elements were also affected by the number of days of bagging. The reduced Ca concentration in long-duration bagging (early bagging) might be due to increased RH around the fruits. Fruits bagged for 55 days recorded an increased content of N, P, Zn, Mn and Fe while fruit calcium concentration was reduced by bagging for 55 days. Anthracnose and stem-end-rot (SER) caused by *Colletotrichum* and *Diplodia* spp. respectively were reduced by bagging in both years through a reduction in contact between disease propagules and fruits. These results indicate that bagging can improve fruit quality by reducing disease, lead to a better appearance of fruit and increase fruit weight and size.

1. Introduction

In almost all fruits including mango, the stage of maturity at harvest is very important as it markedly influences not only ripening and storage but also taste and palatability of the fruit. Consumers seek a mango with a bright, fully developed skin colour without any blemishes, uniformly softened flesh and a fruit with small stone, more pulp and also good flavour and taste with appreciable storage life. Mango fruits when matured on the tree develop most of the above characteristics but such fruits have been associated with environmental hazards such as insect attack, sunburn, wind abrasion, sap spurt at harvest, damage due to hail and pre-harvest disease infection (Oosthuyse, 1997). Bagging may be useful as a means of preventing such problems in mango and can reduce disease and physical damage as well as improve colour at harvest in a number of fruits (Bentley and Viveros, 1992; Byers and Carbaugh, 1995). This approach has been tested to produce high quality unblemished mango fruits in Queensland (Hofman *et al.*, 1997), South Africa (Oosthuyse, 1997) and the Philippines (Bugante *et al.*, 1997).

However, different bagging materials behave in different ways, as has been reported by Ann *et al.* (1998). According to these authors fruit bagging at an early stage was the most effective method to control mango anthracnose disease. The use of different bagging materials did not affect disease controls, although it did affect fruit maturation, colour and °Brix. While some benefits (e.g. reduction in physical damage) could be expected, there may also be negative effects on quality as different days of bagging can delay the development of ripening characteristics of fruits.

The present paper describes the results of experiments on bagging in fruit quality and mineral element content of mango fruit cv. Himsagar, an important commercial cultivar of West Bengal, India.

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2. Materials and Methods

The experiment was conducted at the Mondouri Horticultural Research Station and the post harvest technology laboratory of the Faculty of Horticulture, Bidhan Chandra Krishi Viswavidyalay, Nadia, West Bengal, India during 2006-2008. Study trees of mango cv. Himsager were selected from the orchard at Mondouri (23.5° N latitude and 89° E longitude). The trees were healthy, uniform in size and more than 15 years old. About 250 developing mangoes were tagged and bagged with transparent polyethylene bags at different stages of fruit development: 35, 45, 55 and 65 days after fruit set and a control without any bagging. Then on each sampling date (75, 85, 90 days after fruit set), 10 mangoes were harvested at random from each bagging-date lot of polyethylene-bagged fruits. The fruits were washed and dried at ambient temperature (32°C±1°C), and kept in the laboratory for ripening. Ripe fruits were analyzed for physico-chemical characteristics; bagged fruits were analysed 55 days after fruit set for mineral elements and disease incidence.

Physico-chemical analysis

A. Physical characters. Weight, length and diameter. The weight of ripe fruit was determined using a digital balance and expressed in grams. Length of fruit was measured from the base to the apex and diameter at its widest part, near the shoulder of the fruit, with the help of a vernier caliper. Both were expressed in centimeters.

B. Bio-chemical constituents. Total soluble sugars, total soluble solids (TSS), titratable acidity. Total soluble sugar content was analysed using Fehlings' A and B solution, according to the methods of the AOAC (1996) and expressed as percentage. In this method, for inversion at room temperature an aliquot of clarified and diluted solution was transferred to a flask. 10 ml of HCl (1:1) was added and allowed to stand at room temperature for 24 h. The solution was then neutralized with concentrated NaOH solution and made to volume. An aliquot was taken and the total soluble sugars were determined as invert sugars using Fehling's A and B solution. In determining reducing sugar, acid hydrolysis was not done. Total soluble solids (TSS) content of juice was determined using a hand refractometer and expressed as °Brix at 20°C. Titratable acidity (% malic acid) was estimated by titrating fruit juice (5 ml) to pH 8 against 0.1 M NaOH using phenolphthale as an indicator.

Total carotenoids

Total carotenoids were estimated by the method of Ranganna (1977). Five grams of fresh sample were taken, a few crystals of anhydrous sodium sulphate were added, and then crushed in 10 ml acetone with the help of a pestle and mortar. The supernatant was decanted into a beaker. The process was repeated twice or thrice and the combined supernatant was transferred to a separating funnel out on standing. Petroleum ether (10 to 15 ml) was added in the separating funnel and rinsed; the pigment was then transferred to the petroleum ether phase by diluting the acetone with water or water containing 5% sodium sulphate. The extraction of the acetone phase with a small volume of petroleum ether was repeated, if necessary, until no more colour was extracted. The lower layer was discarded and the upper layer was collected in a 100 ml volumetric flask. The petroleum ether extract was filtered through anhydrous Na_2SO_4 and the volume was made up to 100 ml with petroleum ether. The optical density was recorded at 452 nm using petroleum ether as blank containing 3 ml acetone per 100 ml and expressed as µg 100 g^{-1} pulp. As carotenoids are light sensitive, all steps were performed under subdued light.

Flesh minerals

Dried flesh samples from the ripe fruit (unbagged and bagged 55 days after fruit set) were collected and ground with a mortar and pestle, further dried at 70°C for 2 days, then finely ground in a shatter box. A 0.3-0.5 g sub-sample was digested in 15 ml nitric acid/perchloric acid and analysed by atomic absorption spectroscopy against standards prepared in the same matrix. Nitrogen was determined using Kjeldahl digestion.

Disease incidence

Disease incidence was measured 10 days after harvest. Anthracnose lesions (caused by *Colletotrichum* spp.) on the side of the fruit, and stem end rot lesions (SER : caused by *Diplodia* spp.) at the stem end of the fruit were rated for incidence (percentage of the fruit affected).

Statistical analyses

Data regarding observed characters were statistically analysed by complete Randomised Design and test of significance was carried out following the method described by Panse and Sukhatme (1967).

3. Results and Discussion

Fruit weight, length and diameter

Fruit bagging with polyethylene bags significantly increased the weight of fruit as compared with the control at all stages of fruit harvest (75, 85 and 90 days after fruit set). Early bagging (35 days after fruit set) proved most effective in increasing fruit weight compared to those bagged later (Table 1). It is evident from Table 2 that fruit length

Table 1 - Effect of bagging on fruit weight (g) at different stages of harvest

Bagging (days after fruit set)	Stages of fruit harvest (days after fruit set)		
	75	85	90
35	303.50	322.75	345.00
45	287.50	296.75	319.60
55	244.75	291.20	310.17
65	229.72	259.86	301.87
Control	205.25	229.50	276.02
SEm ±	21.22	28.52	24.12
LSD (P= 0.05)	63.62	85.11	72.42

Table 2 - Effect of fruit bagging with polyethylene on length (cm) of fruit at different stages of harvest

Bagging (days after fruit set)	Stages of fruit harvest (days after fruit set)		
	75	85	90
35	10.75	12.75	13.91
45	10.30	11.50	12.28
55	10.06	11.35	11.94
65	9.95	11.12	11.82
Control	9.40	10.92	11.32
SEm ±	0.425	0.414	0.380
LSD (P = 0.05)	1.241	NS	1.144

increased along with the increase in duration of bagging and also with delayed harvesting. Fruits bagged early (35 and 45 days after fruit set) were almost always longer than those bagged later (55 days after fruit set). However, the increase in fruit length due to polybagging was not significant for fruits sampled 85 days after fruit set. Like fruit length, the diameter of fruit also increased due to polybagging; the increase in diameter was significant at all stages of fruit harvest (Table 3).

Table 3 - Effect of fruit bagging with polyethylene on diameter (cm) of fruit at different stages of harvest

Bagging (days after fruit set)	Stages of fruit harvest (days after fruit set)		
	75	85	90
35	7.77	8.00	8.43
45	7.33	7.71	8.25
55	7.19	7.70	7.95
65	7.05	7.44	7.94
Control	6.91	7.25	7.68
SEm ±	0.172	0.210	0.211
LSD (P = 0.05)	0.521	0.591	0.610

Total Soluble Solids (TSS), total soluble sugar and titratable acidity (TA)

Total soluble solids and sugar contents of fruit were significantly affected by treatment with polybagging (Tables 4 and 5). TSS content of ripened fruit was always higher in those bagged later or not bagged at all. At fruit harvest 90 days after fruit set, TSS content was higher in fruits that were bagged later (at or after 55 days of fruit set)

Table 4 - Effect of fruit bagging on total soluble solids (°Brix) content of ripe mango fruit

Bagging (days after fruit set)	Stages of fruit harvest (days after fruit set)		
	75	85	90
45	9.90	12.20	15.40
55	10.05	13.50	17.30
65	10.40	14.30	17.85
Control	11.90	15.30	16.25
SEm ±	0.161	0.072	0.051
LSD (P = 0.05)	0.483	0.219	0.153

Table 5 - Effect of fruit bagging on total soluble sugar (% of fresh weight) content of ripe mango fruit

Bagging (days after fruit set)	Stages of fruit harvest (days after fruit set)		
	75	85	90
35	4.42	6.85	11.81
45	4.77	6.85	12.57
55	5.02	7.52	13.69
65	5.50	7.81	13.82
Control	6.20	7.97	12.83
SEm ±	0.129	0.061	0.051
LSD (P = 0.05)	0.391	0.180	0.158

than the rest of bagged or not bagged fruits, however TSS content was greatest in fruits bagged earliest (35 day after fruit set). As TSS, total sugar content of fruits was always greater than those that were bagged later (65 days after fruit set) or those that were not bagged at all (control). Fruit acidity decreased with a decrease in bagging period (Table 6). However titratable acid content of ripened fruits did not show any significant variations due to bagging at different stages of fruit development.

Table 6 - Effect of fruit bagging on titratable acid (% malic acid) content of ripe mango fruit

Bagging (days after fruit set)	Stages of fruit harvest (days after fruit set)		
	75	85	90
35	0.250	0.210	0.200
45	0.240	0.220	0.190
55	0.210	0.190	0.180
65	0.180	0.180	0.150
Control	0.170	0.160	0.140
SEm ±	0.012	0.014	0.015
LSD (P = 0.05)	NS	NS	NS

Carotenoid contents

Analysis at the eating ripe stage showed that the carotenoid content of fruit increased as harvesting was delayed from 75 to 90 days (Table 7). Early bagged fruits usually developed less carotenoid upon ripening as compared to those bagged later or to the control. The unbagged fruits

Table 7 - Effect of fruit bagging on carotene (µg/100 g pulp) content of ripe mango fruit

Bagging (days after fruit set)	Stages of fruit harvest (days after fruit set)		
	75	85	90
35	2247.50	4345.50	7782.50
45	2380.00	4773.50	8276.00
55	3565.00	6566.00	8958.50
65	3955.00	7318.50	9168.00
Control	4354.00	8485.50	9768.00
SEm ±	12.95	17.25	19.00
LSD (P = 0.05)	36.79	52.12	57.12

(control) at all stages of harvest developed maximum content of carotene while early bagged fruits (35 days after fruit set) contained minimum carotene upon ripening at all different stages.

Fruit minerals and disease incidence

Mineral element contents of fruits varied due to different days of bagging. Table 8 shows that N, P, Zn, Mn and Fe content of fruits were higher in bagged fruit (55 days bagged) while K and Ca contents were higher in control fruit (0 day bagged) as compared to 55 days of bagging. Bagging did not significantly reduce the incidence of anthracnose and stem-rot (SER) (Table 9) of ripe fruit. Reductions of only 5.5 and 6.0% of anthracnose and stem-end-rot, respectively, were noted in fruits bagged 55 days after fruit set.

Table 8 - Effect of fruit bagging on mineral elements contents of ripe mango fruit

Mineral elements	Days bagged		Significance
	0	55	
N (%) dry weight	0.57	0.74	*
P (%) dry weight	0.12	0.14	*
K (%) dry weight	0.71	0.64	*
Ca (%) dry weight	0.07	0.03	*
Zn (ppm)	22.50	23.33	NS
Mn (ppm)	11.75	12.18	NS
Fe (ppm)	49.00	49.75	NS

Assessment were made at eating soft stage.
NS indicates P>0.1.
* Represent effects significant at P<0.05.

Table 9 - Effect of bagging on fruit disease incidence (percent of fruit affected)

Parameter Incidence (%)	Days bagged	Anthracnose	Stem-end-rot
	0	82.5	77.0
	55	56.5	50.5
	Significance	NS	NS

All assessment were made at 10 days after harvest.
NS indicates P>0.1.

The results of the present investigation clearly demonstrate the benefits of polybagging on the development of mango fruit and fruit quality. Bagging of fruits improved fruit weight and size of litchi (Tyas *et al.,* 1998), banana (Johns and Scott, 1989) and pomegranate (Hussein *et al.* 1994). Bagging can affect fruit size through other effects such as increased relative humidity and therefore reduced fruit water loss (Tombesi *et al.,* 1993). According to Hofman *et al.* (1997), in the present study fruit mass and size may be due to the use of polybag instead of paper bag. The polybagging of fruits delayed the development of ripening characteristics of fruits. However, the extent of the fruit surface coloured yellow was greater with polybagged fruits, which was due to maintenance of a higher temperature inside the bags. In bagged fruits usually day/night temperature fluctuations were reduced, hence the range of minimum temperatures was between 18 and 27°C, which is very close to the optimal temperature range (24-30°C) for the development of Himsagar mango (Mukherjee, 1953; Whiley *et al*., 1989). The total soluble solids and total sugar content of ripe mango were almost always greater in control fruits and those with a shorter bagging period as compared to early-bagged fruits.

Titratable acid content of ripe fruits was greater in early-bagged fruits and acid content of ripe fruits declined as the period of bagging was reduced; minimum acid levels were noted in the control. This is only due to bagging which delayed the development of ripening characteristics of fruits. Similarly, the carotene content in the pulp of ripe mango fruit was at maximum in control and minimum in early-bagged fruits. This is mainly due to fact that polybagging of mango fruits delayed the development of ripening characteristics and therefore there was less development of carotene in fruits that were bagged early in comparison to control. These results are in close conformity with the previous findings of Singha (2002). However, Hofman (1997) opined that fruit quality of mango was not affected by bagging. Fruit mineral (N, P, K and Ca) contents were significantly influenced by bagging. Bagged fruit in the 55-day group had a amount of calcium in comparison to unbagged fruits. Calcium is transported mainly in the transpiration stream (Grange and Hand, 1987), thus tissues with greater transpiration generally have higher tissue Ca concentrations (Witney *et al.*, 1990 b). Increased RH around the fruit can reduce fruit Ca accumulation (Grange and Hand, 1987, Combrink *et al.*, 1995) and bagging of apple fruit has been associated with reducing fruit Ca concentrations and increasing bitter pit incidence (Witney *et al.,* 1991). Similar effects were observed in mangoes bagged for 55 days, although the higher fruit Ca concentration with longer bagging times may suggest a capacity of the fruit to import Ca by a mechanism other than transpiration. Longer bagging times may have caused changes in the surface of fruits that allowed higher transpiration rates and thereby the higher Ca accumulation noted with increased bagging times as described by Hofman *et al.* (1997).

Reduction in fruit disease as a consequence of bagging in a number of different fruits has been noted by Kitagawa *et al.* (1992). In mango, *colletotrichum* infection occurs during fruit development and remains quiescent until fruit ripening (Dodd and Jeffries, 1989). The reduction in anthracnose incidence with bagging (55 days) could be partly due to a reduction in contact between disease propagules and fruit as mentioned by Hofman *et al.* (1997).

In conclusion, bagging of mango can have important commercial benefits as a means to prevent problems like

insect attack, sunburn, sap spurt at harvest and damage due to small hail storms by reducing physical damage of fruit, as well as delaying the development of ripening characteristics of fruits.

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Oleiculture in progress

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Kew words: aging, branching, chilling requirement, cold resistance, flower buds, planting density, triglycerides.

Abstract: The present work evaluates the limits and possibilities of development with regard to the most recent olive-growing techniques in light of up-to-date knowledge of species characteristics. After a brief introduction regarding the productive capacity of olive, the new taxonomic position of the cultivated species and a reorganization of the genus *Olea* is presented in the first part of the work. Examination follows of the assumed stages of domestication, spread (from the Bronze Age until decline in the 6-10th centuries A.D.) and then globalization of the species from the 19th century until the present. The second part addresses the spread of olive to the different continents, environmental limitations to its cultivation and the growth model that distinguishes it from most of the other cultivated woody species. The various problems that arise when olive is cultivated outside its areal of origin are considered, from induction processes to effective chilling requirements, as well as the effects of climatic environment on plant growth and product quality following shifts in areal. The paper concludes with a brief analysis of open questions relative to new models of cultivation.

1. Introduction

Cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) (Green, 2002) ranks 21st among agricultural species worldwide and first among woody fruit species in terms of surface area (9.5 million ha), producing more than 3 000 000 t of virgin olive oil (FAO, 2010) and more than 2 200 000 t of table olives per year (IOOC, 2010).

Virgin olive oil, which makes up slightly more than 2% of total vegetal oil production, accounts for a return in the agricultural sector of more than 22 billion dollars, while the figure for palm oil (30% of vegetal oil production) amounts to a little more than 31 billion dollars (FAO, 2010). Olive cultivation worldwide has grown over the past 20 years by more than 20% in terms of surface area with increases noted on all continents. There has also been a doubling in production, which is more than proportional to the increase in surface area (FAO, 2010). These increases have been due to improvements in cultivation technique in traditional plantings as well as entry into production of new, more rational plantings. The species is in general very efficient in exploiting environmental resources and can produce as much as 2 t ha^{-1} of healthy oil food.

Little more than 50 years have passed since olive first expanded beyond its traditional cultivation areas, and the species has shown to be flexible and adaptable in various agronomic, climatic and environmental situations, able to add value to extensive areas and to face the climate changes currently taking place.

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2. Taxonomy

Cultivated olive is an evergreen bush that can be, with suitable intervention, grown as a tree and it is considered typical of Mediterranean flora. The taxonomic position of the species *Olea europaea* L. has been reviewed (Green, 2002), also in light of the data that has emerged from new technologies of molecular identification (Besnard *et al*., 2002).

The *Olea* genus is divided into three subgenera and in the subgenus *Olea*, sez. *Olea*, the species *Olea europaea* is grouped as a "complex" with potentially infertile forms, compatible for grafting, and characterized by the presence of flavonoid glucosides in plant tissues and fruit.

The *Olea europaea* complex is further divided into six subspecies: *cerasiformis* (Island of Madeira), *cuspidata* (from the south to northeast of Africa and from southwest Asia to the arid zones of the Yunnan and Sichuan in China), *europaea* (Mediterranean basin as far as Mesopotamia), *guanchica* (Canary Islands), *laperrinei* (Hoggar high plain in the south of Algeria as far as al Jebel Marra in the western Sudan), and *maroccana* (southern slope of the Atlas mountains in Morocco). These subspecies can be considered "geographic entities" with notable molecular differences (for example *cerasiformis and maroccana* are polyploid) (Besnard *et al*., 2007 a) but their morphologic characteristics are so similar that in certain cases descrip-

tions can overlap. This fact justifies past imprecision in classifications.

Many entities which in the past were considered to be separate species, including progenitors of cultivated olive such as *O. crysophylla* and *O. ferruginea* (Simmonds, 1976), have been placed within the subspecies *O. europaea cuspidata*.

Based on this classification, the areal of the *Olea europaea* complex occupies three continents: starting from South Africa it crosses the central part of the continent and the Horn of Africa, from Egypt and the Red Sea it reaches the Mediterranean and toward the west into the islands of Macaronesia (Island of Madeira and the Canary Islands), while in the east it passes through Palestine, Syria, Mesopotamia and western and eastern zones of the Himalaya chain as far as southwestern China.

3. Origin and domestication of the species

Due to a scarcity of fossil evidence, it is difficult to determine the geologic period during which this complex *taxa* became defined and began to evolve. It is believed that both a floristic element of African paleotropical origin and the long evolutionary history of this group spanned a large part of the Tertiary (Besnard *et al*., 2009). It seems that the genus *Olea* had its origins in the Oligocene and that numerous *taxa* diversified thanks to catastrophic climatic and tectonic events which were characteristic during the Tertiary (from the great glacial period of the Oligocene to the raising of mountains in eastern Africa and desertification of the Sahara).

A study on the phylogenesis of the *Olea* genus, conducted by Besnard and coworkers (Besnard *et al*., 2007 b) using plastidial DNA and nuclear ribosomal DNA as biological clocks, positions the principal phylogenetic nodes for determining articulations of the genus from the Oligocene (c. 59.2 mya) until the Pliocene (c. 4.4 mya), by which time the main *taxa* of the genus had clearly separated. The desertification of continental Africa seems to be correlated to subsequent differentiation of *taxa* adapted to arid conditions. The new dry environments may have favored establishment of foliar morphotypes in *Olea*. Recurrent segregation and hybridization events could very well have been caused by geographic barriers (e.g. the Sahara) and land bridges.

Probably until the end of the last ice age (15 000-12 000 B.C.) the distribution of olive was prevalently in the area of Africa where desertification led to isolation of Saharan populations (subsp. *laperrinei*). Only later were the southern coasts of the western Mediterranean colonized through the spread of glacial refugia. Thus, spontaneous olive in the western Mediterranean would be of African origin, redistributed as far as the coasts of Spain, France and the Tyrrhenian portion of Italy after the last glaciations (Besnard *et al*., 2001).

The subspecies *O. europaea europaea* is divided into two botanical varieties: *europaea*, which corresponds to the old denomination *Olea sativa* (Weston) and includes the cultivars having seedlings called olivasters; and *silvestris* (Mill.) which corresponds to the old presumed species *Olea oleaster* (Hoffman and Link), spontaneous olive or oleasters.

The wild oleasters forms represent the original postglacial Mediterranean population. In the area between ancient Palestine (Zohary, 1994; Zohary and Spiegel Roy, 1975) and the Caucasus, the earliest plants adapted to the needs of proto-cultivators (around the 5th millennium B.C.) were individuated, utilized and multiplied. Considering the ease of agamic reproduction and longevity of the plant, it is possible that few generations of crosses divide contemporary cultivars from these not-so-distant progenitors.

As colonizers advanced westward, carrying with them plant material and expanding cultivation, they individuated and utilized autochthonous material, progressively diversifying the genetic base of their cultivars and at the same time enriching the local forms.

Generally speaking, significant molecular differences exist between the oleasters of eastern and western zones of the Mediterranean (Breton *et al*., 2006 a) and it is possible to identify separate data groups with a separation line that passes through the Adriatic Sea and the Libyan desert (Breton *et al*., 2006 b). From the cited work, the cultivated genotypes seem rather dispersed which can point to a mixing among the various markers, suggesting repeated attempts at domestication and subsequent crossing among cultivars of different origins. Evidence of these events are illustrated by Hannachi and coworkers (Hannachi *et al*., 2010): comparing plastidial DNA of traditional Tunisian cultivars and wild Tunisian forms, they demonstrated that seven of the 15 cultivars currently under cultivation in that country are of oriental origin, while the others are linked to wild material of Maghrebian origin.

Even if the center of domestication for olive was in the eastern Mediterranean, pre-domestication episodes seem to have occurred more or less at the same time, starting from the 5th millennium B.C., in various areas around the basin, for example on the island of Crete and southern Spain. In these areas "stratifications" have been found with cultivated olive seeds overlaying oleaster seeds, as well as the contrary with "imported" seeds from cultivated forms supplanted by autochthonous forms (Terral *et al*., 2004).

Initially olive was used for different purposes compared to its modern use: it was employed for shade, to produce forage and firewood, and to make tools such as poles and staffs; it took thousands of years of cohabitation to arrive at use of the fruit and oil. When cultivation advanced beyond the phase of simply collecting spontaneous fruits, the proto-farmers decided it was more practical, in a preliminary form of cultivation, to group together the best examples from spontaneous forms through agamic propagation by part of stump. This multiplication technique remained in use until the 1970s in many traditional olive-growing countries of the southern Mediterranean.

4. The spread of cultivation

In the beginning, direct use of the fruits must have been limited since the drupes are very bitter, even when extremely ripe, due to the presence of elevated quantities of oleuropein and other phenolic glucosides, and the technology required to remove the bitterness and for brining was too complex for the means available at the time. However, the oil could be extracted through milling using implements similar to those used for grains, making it available for medicinal use, as fuel, for illumination (both sacred and not) and as an unguent.

The use of olive oil in the diet came later in this plant's history, around the first half of the 2nd millennium B.C., when use of this product spread via sea routes first from the eastern Mediterranean and Aegean Sea toward Greece, then thanks to the Phoenicians along the coast of Africa toward the west as far as (and beyond) the Pillars of Hercules.

It is probable that with this progression, attempts at domestication by the inhabitants of the various zones brought advantages. It should be no surprise that ample deposits of Knossos oil have been found, which testify to the presence of active olive oil commerce in the Minoan period (second half of the 2nd millennium B.C.), nor is transportation of oil from Spain toward Carthage a surprise as cultivation on the Iberian peninsula probably dates back farther than evidence and documentation demonstrate.

At the beginning of the 1st millennium B.C. olive cultivation and oil use had reached the various coasts of the Mediterranean and the type of "domestication" of the plant is revealed through differentiation: the Greeks, as far back as the oldest writings, distinguished the oleaster as κότινος for wood usage from ελαία for oil production.

According to Pliny, the Romans didn't know olive cultivation until the time of Tarquinio Prisco (6th century B.C.) but in the 1st century A.D. oil from the Italian peninsula was exported to the provinces of the Empire. In Rome, oil was principally used for external treatment of the body. As the saying went, "wine on the inside, oil on the outside" (*intus vini fori olei*)[1]

Between the 2nd and 4th centuries the spread of this plant reached its greatest development in the entire Mediterranean region. Oil was distributed free of charge to the plebs of Rome as a food source and sent to the legions in Germany. The trade surrounding oil was so important that remains from the jars and amphorae in the port of Rome of the time left a hill which is today one of the recognized neighborhoods of the city (Testaccio).

5. Globalization of cultivation

With the fall of the Western Roman Empire in 476 AD and loss of control of the sea routes, trade and use of olive in the western Mediterranean declined rapidly. Evidence of introduction of oil from northern Africa in the 6th century can be found (Brugnoli and Varanini, 2005) but from the 7th century sea transport of oil toward Rome ceased, at least in an organized way, to exist. At the same time a period of instability in North Africa began, marking a generalized abandonment of olive cultivation in the region; it did not pick up again until after the Arab conquest around the 10th century.

In Europe, olive oil acquired new importance through the Christian religion, and for which substitutes were not possible (e.g. for illumination of altars, anointing of the sick, use of plant oils during Lent or other periods of abstinence from foods of animal origin). For this reason, cultivation was undertaken in some areas considered marginal in terms of climate for the cultivation of olive (north-central Italy until the Alpine valleys) as a way to at least guarantee the needs for churches and monasteries.

Olive growing zones along the coasts receded with the fall of commerce and only the oldest cultivation areas remained important: Palestine, Syria, and the island of Crete provided oil for Venice and Constantinople while Andalusia furnished Muslim areas.

The olive oil trade was revived in the 11th and 12th centuries thanks to merchants from Genoa and Venice. They supplied monasteries, cities in Italy, France and Constantinople with this precious product needed not only as a food source but also for religious and liturgical purposes, illumination, and soap and wool production.

An intense freeze in 1009 killed off the remains of ancient olive growing on the Italian peninsula along the entire Adriatic and in particular in Puglia. Subsequently this region experienced a notable increase in olive cultivation with new plantings, at the expense of grain cultivation, thanks to the development of trade by the Venetians. In this period Apulian markets were open to traffic from Venice, Genoa and Byzantium, thus progressively establishing this area as an important zone for olive cultivation (Iorio, 2005). Even today Puglia accounts for the most extensive and productive portion of Italian olive production, with more than 20 million trees of 300-500 years of age.

After about 1300, olive oil (for illumination and soap and wool production) became one of the most important products necessary for industrial development in northern Italian cities. As it was not possible to rely exclusively on commercial trade, it became necessary for the economies of many zones to provide incentives for cultivation, also in areas with extreme soil and environmental conditions.

The 15th century represents a turning point not only in the history of olive but also for humanity. In 1453 Constantinople fell, the last remnant of a civilization born with the cultivation of olive, while in 1492 two important events occurred: the fall of Granada, the last vestige of Muslim dominion in Spain, and the landing of Colombo on San Salvador. The interest of Europe shifted westward and with it went the olive, a colonizing plant *par excellence*. Olive was introduced in Spanish colonies, first in Cuba around 1520 and then in California. The original plant material left the port of Seville as seeds or seedlings and so

[1] Plinio, Storia Naturale, XIV, 150.

genetically the material can be considered as Andalusian in origin. The Spanish colonists carried olive with them as they travelled southward from California along the Pacific, introducing it in Peru and from there crossing to Argentina. An olive plant considered to be more than 400 years old exists in the province of La Rioja in Argentina, which would place its establishment at the time of the first European settlements in that region when the capital, Ciudad de Todos los Santos de le Nueva Rioja, was founded (1591) (Fig. 1). During its trip from California to Argentina, the genetic material of olive underwent further selection with identification of cultivars such as 'Mission' (California) and 'Arauco' (Argentina), the 400-year-old tree belonging to this latter.

Fig. 1 - Old picture (dated around 1950) of the oldest 'Arauco' plant in Argentina.

With the spread of colonialism and the need to transfer plant productions able to satisfy industrial and dietary requirements in the colonies, also olive began to be valued, utilized and cultivated in various areas considered to be suitable.

In 1661 Dutch merchants carried olive to South Africa where spontaneous forms of *Olea europaea cuspidata* were already present. Between the end of the 18th and beginning of the 19th centuries cultivated olive, together with *cuspidata*, landed in southern Australia where the two subspecies found a favorable environment, giving rise to a vast phenomenon of spontaneity (Breton *et al*., 2008). The first documented introduction dates back to 1800 when olive officially arrived in Sydney (Spennemann, 1999). From then until before the Second World War, olive from various provenances was introduced (from Spain, Italy, Greece and later Israel). Starting in 1956 there was interest in olive cultivation in China as well, where the plant was known but its cultivation was difficult due to intense summer rainfall.

Currently, the cultivation area of olive is spread in both hemispheres between 45° and 30° of latitude with extension toward the warm-temperate zones of our planet. The agronomic success of this species is based on two fundamental characteristics that distinguish olive from all other cultivated fruit species:

Its particular growth model and flower formation make it easy to manage and the reliability of its production have been determinant for its cultivation since Neolithic times.

It is adaptable to highly variable climatic conditions and its localities of origin for domestication are characterized by different conditions.

6. Environmental limitations

The limits for cultivation of olive are attributable to environmental factors and their annual cycles. Beyond the degree of latitude (45°) for cultivation for this species, plants are potentially exposed to damage from cold temperatures that can compromise production or even the life of the plant, depending on intensity and timing of the event.

Cultivated olive is only moderately tolerant of low temperatures and even if it is suitably prepared for winter cold (acclimatization), it can resist only a few degrees below zero due to a protection mechanism (supercooling) that keeps water in cells in the liquid state down to several degrees below the freezing point (Table 1). The most resistant tissues are the bud meristems (in 'Ascolana tenera' the recorded lethal temperature is -19.3°C) (Fiorino and Mancuso, 2000), so that vegetation severely damaged by

Table 1 - Lethal temperature (°C) as evaluated by differential thermal analysis for various organs in two acclimatized olive cultivars having different resistance to cold: 'Ascolana tenera', very resistant; 'Coratina', poorly resistant

Part of plant	Lethal temperature (°C)	
	'Ascolana tenera'	'Coratina'
Leaves	-14.5 ± 0.2	-11.8 ± 0.2
Shoots	-18.6 ± 0.6	-12.6 ± 0.4
Buds	-19.3 ± 0.6	-13.5 ± 0.4
Roots	-9.1 ± 0.3	-8.6 ± 0.3

From Fiorino and Mancuso, 2000.

intense winter freeze can copiously resprout in the following spring from latent and adventitious buds, in particular on the stump. Differences exist among the cultivars: comparing 21 prevalently Italian cultivars for their resistance to cold, employing three different evaluation methods in leaves and sprouts, the authors (Azzarello *et al.*, 2009) were able to divide the material into three groups in relation to lethal temperature.

With regard to phylogenesis, and considering knowledge of other subspecies such as *Olea europaea* subsp. *cuspidata* which is poorly resistant to cold stress, it is possible that the varieties cultivated today have reached their maximum result possible through genetic pressure applied by humans in terms of advancement toward colder regions.

Furthermore, due to its tropical origin, the species is characterized by a relatively high critical temperature (10-12°C) (Mancuso, 2000), thus pushing cultivation toward northern limits would result in a growing season that is too short. The highest latitude areas with olive cultivation are the alpine lakes in Italy, Istria (Slovenia, Croatia), and the olive-growing area of Odessa (Crimea, Ukraine).

The factors which limit expansion toward the Equator are less evident. Olive is a species that can support high temperature: the lethal temperature for leaf tissues varies from 46 to 50°C depending on the cultivar and can reach 52°C in sprouts (Mancuso and Azzarello, 2002). The greatest limiting factor seems to be the need by flower buds for a period of low temperature in order to pass from an "inductive" phase to that of tissue differentiation and development of the inflorescence. This rest phase is generally divided into two periods with very different thermal needs. In the first phase low temperatures (6-9°C) are needed to remove inhibition for subsequent growth, and in the second warmer temperatures (above 8.5°C) are required to accelerate evolution of the phenomenon. The currently used reference model for olive (De Melo-Abreu *et al*., 2004) is based on that of Richardson to study periods of rest in peach (Richardson *et al*., 1974). This model considers the period from 1 October to 31 January useful for overcoming chilling need, with 7.3°C being the optimal temperature. Temperatures from 0 to 18.5°C can also be considered acceptable with a weighted effect of their distance from the optimal temperature, similar to the Richardson model. The authors terminate calculation of the chilling units on 1 February, as they consider that for their study area (Cordoba and Mas Bové Reus Terragona in Spain, Elvas and Santarém in Portugal) by that date the needs of olive for cold should already be satisfied. The authors underline that temperature is not the only factor toward which olive is sensitive and thus the data needed for this calculation should be verified every time there is a change in cultivation zone or variety.

Interest in the effect of low temperatures for normal development of flower buds is relatively recent: only in the 1950s was it proposed that the amount of flowering is in some way linked to the duration of low winter temperatures (Hartmann, 1953; Hartmann and Porlingis, 1957). In the beginning researchers (Hackett and Hartmann, 1964) believed that the role of low temperature was more incisive and able, together with other environmental factors, to influence flowering processes from the first phases of induction, but later it was demonstrated (Rallo and Martin, 1991) that its role is limited to development subsequent to the induction phase.

There are however ample areas of research that have only barely been considered. For example, in 1975 it was noted in a study (Hartmann and Whisler, 1975) that: 1) by applying a suitable period of cold, flowering can be stimulated in any time of the year (this somehow confirms the theory of "aging" of apical meristems that when mature produce buds able to directly develop flowers) (Fiorino and Marone, 2010); 2) there are ample differences between cultivars in regard to their response to various quantities (constant or variable) of low temperatures (varietal differences represent the weak point of all experimentation in this field); and 3) thermal thresholds of response seem extremely various among cultivars themselves as evidenced by the behavior of local cv. Mission which can, by flowering continuously throughout the cool summer typical of the California coast, lead progressively to a modest number of inflorescences.

In 1983 a work was published (Denney and McEachern, 1983) aimed at improving understanding of responses of this species to various environments. The results were obtained by elaborating temperature data (October-May) from 15 olive-growing stations to determine the effect of the "cold", measured as the capacity of the olive plant to flower in the subsequent spring, when two conditions have been met:

1) active growth has concluded;
2) daily temperature trend does not exceed an average of 12.5°C.

The greatest interest with regard to this study concerns the concept of "effective chilling" which indicates, for regular flowering to occur, the number of days when the average temperature must not drop above 12.5°C. In order to satisfy the thermal needs of olive, 70-80 days of "effective chilling" are necessary, thus setting geographic limits for the species.

A comparative analysis of the thermal conditions of some geographical zones at the warmer limits for cultivation (Ico, Peru and Caborca, Mexico) and where olive growing is undergoing development (Gran Chaco, Argentina) (Ayerza and Sibbett, 2001) points out that often in "new", "warm" olive-growing areas the number of days for wintering are fewer than those considered necessary (70-80 days). For example, at Ico (Peru) the possibility for wintering does not exist as average daily temperatures are never below 12.5°C, although there are cultivars that produce well enough in the area to allow commercial plantings. The area is characterized by a lack of rainfall despite frequently cloudy skies, making it possible to control growth through drastic reductions in irrigation water which oblige the plant to reduce or interrupt vegetative growth, despite year round temperatures that are relatively high. The authors of the study admit that the reasons for this

behavior are not clear and hypothesize that in the autumn-winter period, as the sky is almost constantly overcast, the thermal lows needed for the buds are attained, although perhaps they wouldn't be in sunny areas.

Sensitivity to different thermal thresholds or chilling needs to bring about flowering is appearing in new plantings, in areas considered to be homogeneous with consistent seasonality, but where there are specific zones with cold periods of different duration and intensity. For instance, this can be the case in the Mediterranean basin where notable differences are recorded for Florence (central Italy), Seville (southern Spain) and Cairo (Egypt).

The millennia-long history of olive has made it possible to identify local cultivars perfectly suited to specific environments, thus creating varietal standards able to adapt themselves to the thermal trends of that particular area. In recent olive-growing, cultivars famous in a specific territory have often simply been used elsewhere, although this has led to unsuccessful attempts generally due to a lack of adaptability to the relative high winter temperatures of the new environment. Examples of this are the negative results obtained from the transfer of 'Frantoio' (cultivar from Tuscany) to southern coasts of the Mediterranean or warm areas of the planet.

This sensitivity of the plant to specific thermal requirements for flowering confirms the fundamental role of temperature in all aspects of the life of olive, from the progression of phenological phases to the biochemistry of the oil.

There are notable differences among Mediterranean germplasm with regard to the effective chilling requirements of autochthonous material which do not necessarily depend on where the material was selected. Thus, two varieties selected in similar environments and at approximately the same latitude, for example 'Arbequina' and 'Frantoio', have demonstrated opposing behaviors when cultivated in warm areas: the former flowers copiously and early, while the latter rarely flowers much and often not at all.

In olive, the effect of low temperature is more complex than in temperate species which are often referred to: for some varieties the "chill" that results from diurnal temperature variation is sufficient to permit normal flowering (e.g. in 'Arbequina') and the thermal threshold of chilling must be rather elevated since nocturnal temperatures of less than +2 to -1°C markedly diminish flowering under controlled conditions (Malik and Bradford, 2009).

7. Current trends

Over the course of the millennia of expansion (4th-2nd millennia B.C.) and cultivation (1st millennium B.C. - 2nd millennium A.D.) the techniques for growing olive remained essentially unchanged, just as the needs of agriculture to have long-living, hardy, easily managed plants able to produce oil for nutrition, illumination, industries (textile and mechanical) and other purposes did not change over time. It was only in the 19th century that olive-growing began to gain advantage from technological development, in particular with regard to oil extraction, while agronomic techniques remained anchored in acquisitions and requirements from the past. Research pertaining to olive-growing was only blandly affected by the innovative spirit that invested agriculture and in the first half of the 20th century knowledge based on millennia-old, empirical observations persisted or models and concepts were adapted that pertained to other cultivated woody species, for example fruit-bearing species from temperate zones.

At the turn from the 19th to 20th centuries, in Europe olive oil was considered a strategic food but it was only in the middle of the 20th century that the true importance of this product for human nutrition was understood in terms of its nutritional, organoleptic and functional profile. With economic and geographic expansion of olive cultivation in the second half of the last century came awareness of the peculiarity, potentiality and lack of scientific knowledge about this ancient species.

Olive possesses all the fundamental requisites to become a modern crop: good productivity (more than 2 t ha^{-1} per year of oil), very early entry into production for plantings that are well positioned in terms of water and nutritional availability (on average the third year after planting), good adaptability to diversified environments and availability of light, soil and other resources, good availability of plant material that can be obtained using new techniques (mist-propagation and micropropagation), and great flexibility of plant material for adaptation to various breeding systems, all depending on the various destinations of the product (for oil or the table) and in relation to the needs of the market.

8. Architecture of the plant

Olive owes its agronomic success to the longevity of plantings, simplicity of its constitution and reconstruction of the canopy, and predisposition for flowering.

In nature, the plant grows as a bush with a stump rich in vegetative meristems able to produce suckers that can form trunk-like structures able to survive for hundreds of years; it can grow to have canopy heights and diameters of 10-12 m.

The olive model is not greatly described in literature. Each vegetative meristem on the stump (or positioned plantlet) can give rise to a sprout with acrotonous, orthotropous attitude and continuous growth that will form the vegetative axis. Early on, this sprout is poorly lignified, slender in relation to its height and tends to curve if not supported. At its point of curvature (the new tip of the branch), another vegetative meristem grows and repeats the cycle, while the distal portion of the original branch continues his growth in a lateral direction.

With lignification and subsequent radial growth, the insertion angle of the two consecutive, opposing sections attenuates and the structure takes on the form of a single trunk resulting from fusion of the segments (Fiorino *et al.*, 2012).

The originally-vegetative apical meristems, over time and with growth, mature, change in function, lose their orthotro-

pous characteristics, become plagiotropous and take on reproductive functions ("aging") (Fiorino and Marone, 2010) with subsequent formation of fruit-producing vegetation.

This particular type of growth and shift to production makes olive a very flexible plant as it can be grown either with a central axis and short branchlets arranged at various heights or with varying vase-like forms, obtained via subsequent vegetative axes, having or not a central trunk. Having these characteristics, the parts of the canopy destined to support the vegetation are semi-permanent structures that periodically need to regenerate the growth-aging-flowering cycle typical of olive branches. This growing habitus simplifies pruning principles and operations.

9. Induction and differentiation

Growth and lengthening of the branch in subsequent years is almost completely delegated to apical meristem activity, creating a continuous linear structure (branch-limb) with persistent (three years), opposite leaves and lateral flowering that allows further apical growth. The branch-limb complex is made up of a succession of nodes generated by an evolving meristem (vegetative vs. reproductive) that will determine bud functions. The growth-aging process continues until a progressive weakening leads to the loss of the apical meristem or until itself transforms into a flower (Fig. 2).

Fig. 2 - Terminal bud transformed in floral grape in cv. Koroneiki (Photo E. Marone).

Two types of buds coexist at each node. The main, most evident ones that can remain on the plant for no more than two vegetative cycles, and at least two accessory buds positioned above the main one (Fig. 3). These accessory buds are poorly visible and often covered after their formation by cortical tissues (and therefore also called hidden buds) of the growing branch to the point of not even being considered (Lavee, 2007), and are destined to become latent and able to burst many years after their formation.

Fig. 3 - Node showing the two main buds producing floral grapes and in upper position the two accessory buds (Photo E. Marone).

The evolutionary phases of a flower meristem are not distinguishable to the naked eye before the burst of the buds (Barone and Di Marco, 2003; Andreini *et al*., 2008) and sometimes, even under morpho-anatomical analysis, some early manifestations of differentiation are ambiguous and difficult to interpret (Troncoso, 1966).

Histological and histochemical indications point out that: a) there are histochemical differences in the formation and development of lateral buds in different genotypes (e.g. 'Leccino' and 'Puntino'); b) there are differences in zeatin levels in buds of the same cultivar (e.g. 'Leccino') taken from plants with different fruit loads and these hormone level differences start early, from the month of July (Andreini *et al*., 2008); c) there are differences in development (timing and forming structures) among homologous buds removed from rising branches or identified in peripheral parts of the canopy and these also start early, from the month of July (Fabbri and Alerci, 1999).

It was believed (Lavee, 2007) that in growing sprouts the principal buds of originally mixed function could shift toward flower formation only when a specific sequence of events occurred, which are successive (preinduction-confirmation), temporally separated and controlled by endogenous and exogenous factors.

More recent research has indicated the primary role of apical growth in the formation of fruit-bearing vegetation and in the evolution that is determined by growth. In order to reach adequate levels of aging, in relation to the cultivar, buds with defined function - flowers for the principal buds, vegetative for the accessory buds - form along the

lengthening sprouts (Fiorino and Marone, 2010; Marone *et al*., 2013).

In this way, and possible due to an adequate intensification of cultivation, the amount of annual growth of mature branch-limbs increases leading directly to an increase in the number of flower clusters and thus fruit load on the branch. The fruits, due to their weight, pull the vegetation downward forming fruit-bearing cascades that are particularly suitable for mechanical harvesting by horizontal shakers (Fiorino *et al*., 2010).

10. Adaptability

Due to olive's adaptability to xeric environments, currently expansion of cultivation takes place with success mainly in warm-temperate dry zones below 30-35° latitude in both hemispheres: these areas, where land is available, are characterized by short winters, early-spring temperature rise and hot, sunny summers, all factors which affect the quantity of growth, progression of phenological phases and oil characteristics as particularly influenced by temperature, according to results presented in the literature.

In a study carried out at one location (Montepaldi, Tuscany, 43° 40' N Lat., 11° 09' E Long., 266 m a.s.l.) to evaluate the behavior of cultivars selected over time from areas having different climatic conditions (Mancuso *et al*., 2002), analysis of the relationship between variations in annual climatic conditions and development of phenological phases revealed the following roles played by temperature:

a) in the determination and evolution of the various phases together with different behavior of cultivars coming from different areas. Cultivars 'Coratina' and 'Carolea', selected from southern Italy where spring temperatures are warmer, have more accelerated bud burst and phenological phases up until fruit set compared to cultivars ('Moraiolo' and 'Leccino') selected in the cool, test area; and
b) in the appearance of phenological phases in relation to average increase in temperature and duration of insulation. From a data pool pertaining to five cultivars (the four previously mentioned plus 'Picholine Languedoc') it was found that an increase of 0.5°C leads to an anticipation of flower bud burst by more than three days, with notable effects from the time of pit hardening. In addition, an increase in average insulation seems to increase the speed with which these phenological phases progress.

The influence of spring temperatures on the date of flowering in different cultivars was confirmed by De Melo-Abreu and coworkers (De Melo-Abreu *et al*., 2004). More recently, Orlandi *et al.* (2012) found that pollen release was generally determined by meteorological factors in the period before flowering, with effects on both pollen amounts and timing of flowering (early or delayed).

The role of temperature in oil composition is more extensive. Independently in each cultivar, temperature trend during fruit growth and maturation influences the ratio among fatty acids of the triglycerides in the oil (Fiorino and Ottanelli, 2003; Marone *et al*., 2003) with a reduction of the percentage normally expected for oleic acid in years characterized by prolonged periods of elevated temperature. With increased average seasonal temperatures (expressed as thermal sums, GDH, with 10°C threshold) (software for the calculation of GDH with variable thresholds was elaborated by Marone, 2003), the percentage of oleic acid content drops and is substituted by increases in palmitic and linoleic acid percentages.

Employing a collection of Italian germplasm, with plants grown in the same environment (Mirto, Calabria) and with adequate agronomic techniques, and through analysis of the variations between levels and ratios of the three principal fatty acids (palmitic, oleic, linoleic) and the thermal sums values of GDH for the relative years, it was possible to measure the existing regression between thermal sums and levels of oleic acid percentages in the oils obtained from the cultivated accessions (Fig. 4) (Lombardo *et al*., 2008).

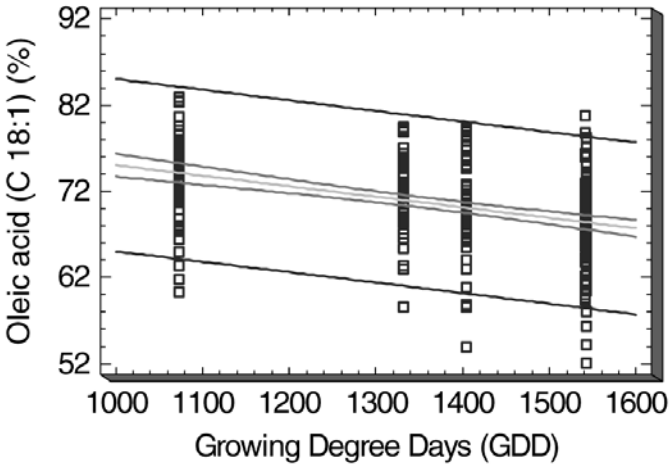

Fig. 4 - Regression between the % content of oleic acid in the TAGs of different cultivars and GDD (population 188 cultivars in total with at least two analytical data in the five years; p≤ 0.01 and r=0.473). On the left, the coolest year (2005), on the right, 2003 (the warmest, practically superposed with 2001) (From: Lombardo *et al*., 2008).

Each cultivar constantly adapts its triglycerides composition, modifying equilibriums and increasing palmitic or linoleic acid (Lombardo *et al*., 2008). The graph shown in figure 5 illustrates the division of a population of cultivars into three clusters separating samples with different characteristics. The first, the most numerous, groups the cultivars that compensate for the reduction in percentage of oleic acid with linoleic acid and subordinately palmitic acid (cv. Canino, Nera di Gonnos, Tonda di Cagliari and Moraiolo). The second group reacts in the opposite manner (cv. Raja sabina and Moraiolo T. Corsini), while only 5% of the tested population is composed of stable triglycerides (cv. Nocellara messinese). The intensity of the response to temperature changes can be such that the oil from some cultivars

approaches or exceeds the limits established by law in some countries with regard to triglyceride composition.

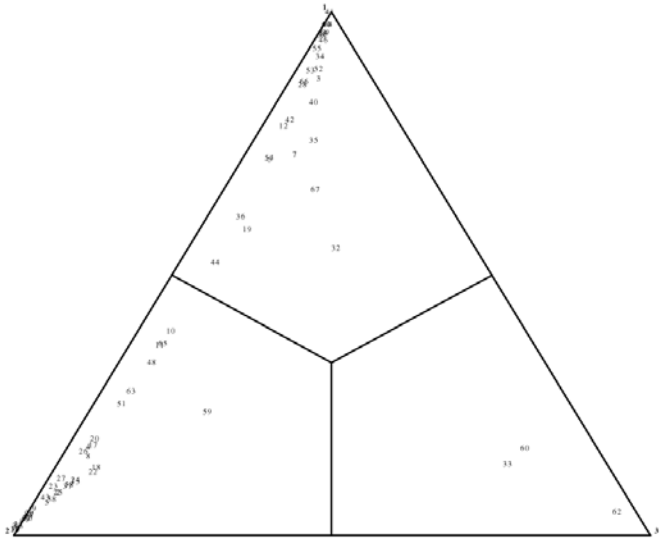

Fig. 5 - Fuzzy clustering in a ternary plot (fuzziness f= 2.25), utilizing the absolute value's differences for the two compared years between 69 cultivars for saturated, monounsaturated and polyunsaturated FAs (From: Lombardo *et al*., 2008).

Working in the province of Rome, Italy (41° 53' N Lat., 12° 14' E Long., 30 m a.s.l.) on 'Arbequina' and 'Arbosana' plants grown as hedges under superintensive cultivation, the acidic composition of the oil was well balanced and characterized by elevated amounts of oleic acid and low or very low levels of linoleic acid in both cultivars (Marone *et al*., 2009). However, when the same varieties were grown in warm, dry valleys in the northwest of Argentina, with a prolonged growing period and high temperatures during the growing season, the acidic composition was modified: there was a drop in oleic acid and a rapid rise in level of linoleic acid (Rondanini *et al*., 2011).

Due to this sensitivity to high temperatures during fruit growth and maturation, the need to review the limits for some parameters in the oil, as prescribed by international laws intended to verify the genuineness of the product, becomes necessary with the extension of olive growing in warm areas or the marketing of local products that had previously been destined for self-consumption. Unadulterated productions can, for example, present not only variations in the ratios among triglycerides (often the amount of linoleic acid exceeds the allowed limit of 1%), but also alterations with regard to the phytosterols, with increases in particular in campesterol levels beyond established limits and which could indicate an addition of seed oils to the olive oil.

There have been few observations of the result of transfer of cultivars from warm to cooler zones. In this case, an opposite phenomenon has been noted with an increase in oleic acid percentage (Fiorino and Marone, unpublished data); it is possible that special characteristics of some productions from cool olive cultivation zones derives precisely from the specific interactions between some cultivars and the various climatic zones.

11. Open questions

New plantings require the transformation of current cultivation techniques, which are still tied to millennia-old traditions. This shift calls for not only radical cultural changes, but also notable investment for establishment of new or modification of existing plantings as well as for machinery. The objective of new plantings is to produce oil, with a lengthening of the production chain from the fruit to the finished product - extra virgin olive oil - which remains unique among other vegetal oils on the market for the presence of complex antioxidants (i.e. polyphenols) that derive directly from this plant's origins.

For researchers and technicians who direct their efforts toward globalization of cultivation, the weak point remains the modest amount of basic knowledge available in order to respond to questions that arise regarding varietal adaptability to different environments, regularity of production and canopy management.

In particular, with regard to the spread of new intensive or superintensive olive-growing models (terms linked to the elevated number of plants per hectare), there are a series of questions: What is the productive lifespan of orchards? What are the characteristics of the product? What is the varietal platform?

The reduced productive lifespan of orchards represents the greatest difficulty to overcome for the spread of this model in countries where olive-growing is deeply rooted in culture and history. Today, plantings last only a few years: about 10 for peach, 15 for apple and pear, perhaps 20 or a bit more for olive, with plants having performed their economic duty in this period of time.

As for the characteristics of the product, research has pointed out that it depends more on cultivar/environment interaction than on the growth system. Initial data seem to confirm that, in any case, the chemical and sensory characteristics of the oil do not change in intensive or superintensive plantings (Marone *et al*., 2009).

The varietal platform suitable for superintensive plantings is made up of few cultivars typical of specific areas ('Arbequina' and 'Arbosana', Spain; 'Koroneiki', Greece). Growth and productivity characteristics are known for the new planting models (Rallo, 2006; Tous *et al*., 2003) as well as the characteristics of the product (Marone *et al*., 2009). Novelties, such as Tosca 07® and Chiquitita® (Redacción Olint, 2007; Rallo *et al*., 2008), have been more recently introduced in new plantings and still need to be better defined agronomically.

The longevity and vitality of olive, and the marginality of its cultivation and production have penalized genetic improvement of this plant: it was undertaken unconsciously in the past but has been more targeted in the last century.

The ideotypes must combine different characteristics (Fiorino, 1999). In addition to growth habit, fructification,

and resistance to biotic and abiotic stress, they have to respond to precise composite characteristics for oil which indicate the nutritional value for humans and are by now used internationally for marketing of the product.

Despite the existence of a broad genetic base, with a total of more than 1200 cultivars in germplasm collections (Bartolini, 2008), there is little reliable information available about genetic determination of traits and their heredity (Bellini *et al*., 2003) and this lack makes it difficult to choose the genetic material to use for crossing programs. A recent review (Rugini *et al*., 2011) about the major needs for genetic improvement of olive, indicated systems and aim to achieve different goals in this species, but at present it often ends up being based simply on the phenotypic behavior of parental lines. Also variability in the triglyceride and unsaponifiable fraction in descendents from crosses with the same parents was wide (Tables 2-4) and sometimes with percentages of some fatty acids outside normal values (Fiorino, 2001). The Table 2 reports the values for fatty acids, polyphenols and tocopherols in oils obtained from eight genotypes of open-pollinated 'Arbequina' grown in the same environment. The lineage is characterized by great variability in acidic composition, above all for linoleic acid contents.

It has only been in the past ten years that indications regarding the technological characteristics of the various germplasm collections are present in terms of the qualitative and organoleptic characteristics of the oil (Rotundo and Marone, 2002). Furthermore, characterization of the oils produced by single cultivars in specific environments has begun in this past decade as well (Cimato *et al.*, 2004; Di Vaio, 2012).

New plantings call for control of canopy dimensions, and as most varieties selected over the millennia are very vigorous, new input has come from selection of clonal rootstocks able to influence growth and vigor in olive. By using rootstocks from specific varieties ('Tosca 07® and 'Leccino dwarf') vigor and architecture have been modified in plants of 'Cerasuola' highly vigorous and 'Bianco-

Table 3 - Values for the most important fatty acids in oil from crosses of 'Arbequina'

Fatty acids (%)	Genotypes		
	127*	129*	B28**
C 16:0	13.7	11.7	19.6
C 16:1	0.9	0.8	2.4
C 18:0	2.1	2.3	2.1
C 18:1	66.1	76.6	50.2
C 18:2	14.7	6.3	23.3
C 18:3	1.0	0.8	1.2

* no. 127 and no. 129 = 'Arbequina' x 'Aggezi Shami'.
** B28 = 'Arbequina' x 'Picholine Languedoc'.
From Fiorino, 2001.

Table 2 - Oil characteristics of genotypes obtained from open-pollinated 'Arbequina' (Ghiza, Egypt)

Fatty acids (%)	Genotypes							
	16	52	56	61	67	68	94	105
C 16:0	10.7	14.2	14.2	16.2	19.3	14.9	15.7	18.6
C 16:1	1.0	0.6	1.2	2.2	3.4	2.0	2.1	3.4
C 18:0	2.5	2.1	2.3	2.2	1.8	1.7	2.5	1.7
C 18:1	75.3	66.7	50.7	60.6	42.0	45. 9	61.1	58.5
C 18:2	8.5	14.2	29.3	16.6	31.7	33.3	16.6	15.7
C 18:3	0.7	0.9	1.3	1.2	0.9	1.2	1.0	1.0
Tocopherols (mg/kg)	416	168	219	366	286	347	91	420
Polyphenols (mg/kg)	34	165	67	51	77	91	50	101

From Fiorino, 2001.

Table 4 - Oil characteristics of genotypes obtained from controlled crosses of 'Manzanilla' x 'Picholine Languedoc'

Fatty acids (%)	Genotypes					
	A2	A5	A4	A14	A18	A19
C 16:0	12.0	10.9	13.7	16.6	9.9	16.1
C 16:1	0.9	1.2	1.9	1.9	0.9	2.1
C 18:0	2.5	2.1	2.3	1.9	2.1	2.1
C 18:1	68.6	75.0	64.9	63.9	72.0	58.0
C 18:2	13.7	8.8	15.1	13.7	12.8	19.5
C 18:3	0.8	0.7	0.8	0.9	0.9	1.3
Tocopherols (mg/kg)	173	185	215	177	214	318
Polyphenols (mg/kg)	169	-	286	-	140	305

From Fiorino, 2001.

lilla' low vigorous (Caruso *et al*., 2012). These results are very promising, even if they must be verified in the field and in different environments.

12. Conclusions

As with other woody fruit trees (e.g. *Citrus* spp.) and prevalently herbaceous species (e.g. corn, soy, sugarcane, manioc), olive is rapidly expanding its territory into new areas, which in turn permits the latent capacities of this species to be expressed to an equal or greater extent compared to that possible in the species' areas of domestication and millennia of cultivation.

Olive seems able to gain added value in some temperate-warm areas of the planet where otherwise unutilized desert zones could offer ample space. In these areas it would be possible to control growth and fructification through the water stress inherent to the climate as well as temporal space during the annual growth cycle to satisfy the modest chilling requirements of some cultivars.

The current geographical limit toward the equator (30° Lat.) does not seem insurmountable and, in particular conditions, the margins for maneuvering with regard to enlarging the areal still seem quite wide. The limits of this development are related to a lack of some background knowledge and uncertainties in interpretation of research data, especially with regard to control mechanisms for flowering, flower formation (inductive phase), and flower development (chilling requirements).

Olive oil has special characteristics that make it not only a food and condiment, but also a product able to protect the human organism from dysfunctions and pathologies, thanks to the presence of a number of components, and thus it plays an important role in a balanced diet.

Olive has a particular capacity to respond to changes in its environment (especially vegetation and fruits with regard to temperature) and greater development in warm cultivation areas is possible but first more knowledge is needed regarding genotype/environment interactions of varieties suitable for cultivation in these zones to maintain olive oil as a preferred source of vegetal fats.

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Mechanical harvesting of oil olives by trunk shaker with a reversed umbrella interceptor

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Key words: mechanical harvesting, *Olea europaea* L., '*Ortice*', '*Ortolana*', oil quality, trunk shaker with reversed umbrella.

Abstract: Trunk shakers are primarily used for the mechanical harvesting of oil olives in intensive orchards. The objective of this trial was to determine the efficiency of mechanical harvesting of olives with a self-propelled trunk shaker with a reversed umbrella interceptor (model F3, SICMA, Catanzaro, Italy), from adult trees of two autochthonous cultivars, 'Ortice' and 'Ortolana', growing in southern Italy with 6 × 6 m spacing and trained to the vase system. The main characteristics of the trunk shaker were: an engine power of 77 Kw (105 CV), a very-high-frequency vibrating head (1800-2000 vibrations/min), a self-braking system and a 6-meter diameter umbrella opening. The worksite consisted of two workers one for maneuvering the harvesting machine and the other for handling the olives. Mechanical harvesting was carried on 30 November 2006 when the fruits of 'Ortice' and 'Ortolana' had a weight and detachment force around 2.8 g and 3.1 N and 3.8 g and 4.6 N, respectively, and the fruit drop was around 14% and 10%, respectively. Both cultivars had a good production (26.06 and 21.18 kg/tree). The mechanical harvesting yield (percentage of mechanically harvested olives) was very high, reaching values around 97% in both cultivars. Moreover, the low number of workers, the reduced time for the operation (2.5 min/tree), the good yield/tree and the high quantity of harvested fruit allowed a very high work productivity to be obtained: around 302 kg/h/worker for 'Ortice' and 246 kg/h/worker for 'Ortolana'. The quality of the oils extracted from the harvested olives met the requirements set by European law for extra virgin olive oils. The results indicate that the use of a trunk shaker with a reversed umbrella can be an efficient solution for mechanical harvesting of the 'Ortice' and 'Ortolana' cultivars in southern Italy.

1. Introduction

Harvesting is one of the most important operations of the whole cultivating cycle in olive production, both in order to obtain high quality oils and to reduce costs (Tombesi, 1990; Famiani *et al*., 1998; Cicek, *et al*., 2010; Ferguson *et al*., 2010). The aim of this trial was to evaluate the efficiency of a trunk shaker with a reversed umbrella interceptor for the mechanical harvesting (Visco *et al*., 2008; Farinelli *et al*., 2012 a, b) of two autochthonous cultivars, 'Ortice' and 'Ortolana', in southern Italy.

2. Materials and Methods

The experiment was carried out in 2006 in a commercial olive grove, belonging to the "Uliveto" farm, located in southern Italy (41°15' N, 14°38' E) (Province of Benevento). Adult trees of two autochthonous cultivars, 'Ortice' and 'Ortolana' (Di Vaio *et al*., 2013), trained to the vase system and planted at a spacing of 6 x 6 m were studied. The olive grove had a slope of less than 3% and was drip irrigated. Pruning was carried out annually and fertilization and pest management were carried out according to local standard practices. For the mechanical harvesting, carried out on 30 November, a self-propelled machine, "F3" model with three traction wheels and a reversed umbrella interceptor manufactured by SICMA (Catanzaro, Italy), was used (Fig. 1). The main characteristics of the trunk shaker were: an engine power of 77 Kw (105 CV), a very-high-frequency vibrating head (1800-2000 vibrations/min), a self-braking system and a 6-m diameter umbrella opening. The work force consisted of two workers, one for maneuvering the harvesting machine, the other for handling the olives. During drupe maturation, periodically on three samples of 100 olives per date, the following parameters were determined: olive detachment force, coloration (Jaén index between 0 and 7), fresh weight, pulp firmness (with a manual penetrometer with a 1.0 mm diameter plunger on the equatorial zone of fruit) and oil content (determined using a Soxlhet extractor). On four trees per cultivar, at the

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Fig. 1 - Mechanical harvesting of olives with a trunk shaker with a reversed umbrella interceptor.

beginning of ripening, eight small branches were labeled (two per cardinal point) and the fruit was periodically counted up to harvesting time in order to estimate fruit drop. Mechanical harvesting was carried out on 10 trees/cultivar and the drupes were weighed. After harvesting, undetached olives were harvested by hand and weighed. The ratio between mechanically harvested olives/total olives on the canopy, expressed as percentage, was used to determine the mechanical harvesting yield (%). The trunk cross-sectional area (at about 0.5 m above the ground) and canopy width (W), height (H) and volume [Volume = $((W/2)^2 \times 3.14 \times H) \times 2/3$] were measured/calculated on each of the harvested trees. Work productivity was calculated and expressed as the amount of harvested olives/h/worker. In both cultivars, after mechanical harvesting, 100 kg of olives were collected and micro-milled to obtain two samples of mono-variety oils, on which free acidity, peroxide number, spectrophotometric indices and sensorial characteristics (by panel test) were determined.

Data were submitted to analysis of variance (ANOVA) using MSTA-C software and mean separation was performed by the Multiple Range Duncan test at the 5% significance level. Moreover, the standard errors (SE) of the means were also calculated.

3. Results and discussion

During maturation, the cv. Ortice was resistant to drupe detachment, which decreased at the beginning of November until reaching 309.17 g at harvest time. The cv 'Ortolana', instead, followed a rather constant course, and at harvest time reached 455.83 g (Fig. 2 and Table 1). Pulp firmness decreased constantly during maturation, reaching

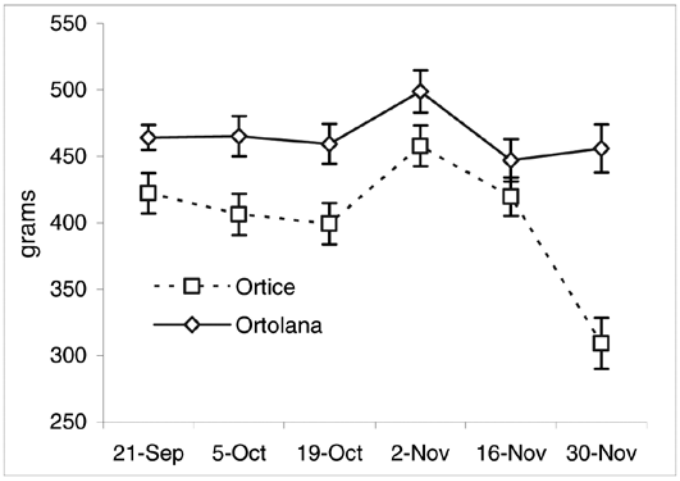

Fig. 2 - Evolution of olive detachment force during maturation (mean ± standard error).

Table 1 - Characteristics of trees and fruit at harvest, machine efficiency and work productivity (mean ± standard error)

	cv. Ortice	cv. Ortolana
Olive weight (g)	2.79 a±0.06	3.77 b±0.01
Olive detachment force (g)	309.17 a±19.30	455.83 b±18.20
Olive detachment force/olive weight (N/g)	1.11 a±0.08	1.21 a±0.09
Pulp firmness (g/mm^2)	267.00 a±5.11	231.58 b±0.45
Drupe coloration - Jaèn index (0-7)	2.10 a±0.05	4.35 b±0.17
Fruit drop (%)	13.95 a±2.27	10.36 a±2.49
Canopy volume (m^3)	47.34 a±2.35	56.31 b±1.56
Trunk cross section area (cm^2)	1587.31 a±337.96	613.43 b±172.04
Total olive yield per tree (kg)	26.06 a±7.72	21.18 a±5.02
Olives mechanically harvested per tree (kg)	25.17 a±7.21	20.49 a±5.03
Productive efficiency of tree (kg/m^3)	0.37 a±0.17	0.25 a±0.13
Mechanical harvesting yield (%)	96.58 a±1.15	96.74 a±0.88
Work productivity (kg/h/worker)	302.04 a±18.01	245.88 a±14.10

In each row, means with the same letter are not significantly different by Duncan multiple range test (P<0.05).

values of 267.00 g/mm^2 and 231.58 g/mm^2 for 'Ortice' and 'Ortolana', respectively (Fig. 3 and Table 1). At harvest, the accumulation of oil in the drupe was 26.09% in 'Ortice'

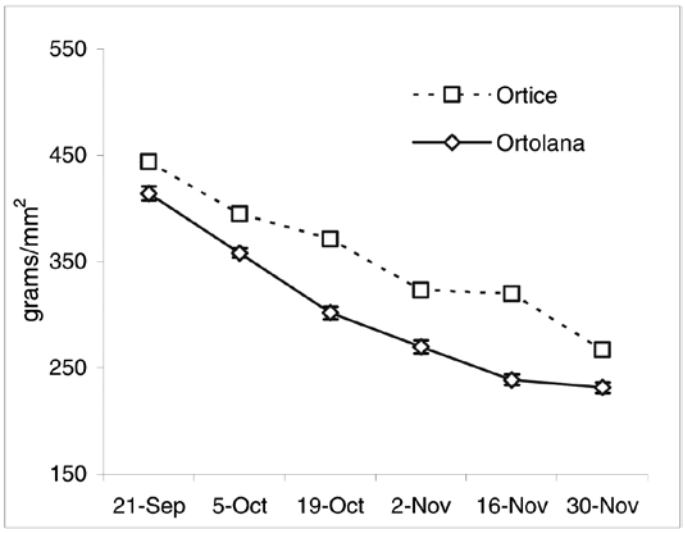

Fig. 3 - Evolution of pulp firmness during maturation (mean ± standard error).

and 16.51% in 'Ortolana' (Fig. 4). Olive pigmentation, scored according to the Jaén index, differed for the two cultivars: 2.10 in 'Ortice' and 4.35 in 'Ortolana' (Table 1). Both cultivars had good production (26.06 and 21.18 kg/tree) (Table 1), and the productive efficiency was 0.37 and 0.25 kg of olives/m^3, respectively (Table 1). The canopy volume of the trees was 47.34 m^3 for 'Ortice' and 56.31 m^3 for 'Ortolana'. Pre-harvest fruit drop increased during the entire period, reaching values of 13.95% for 'Ortice' and 10.36% for 'Ortolana' at harvest time (Fig. 5 and Table 1). At harvest fruit weight was 2.79 g for 'Ortice' and 3.77 g for 'Ortolana' (Table 1). The ratio between the olive detachment force and weight decreased throughout the ripening period and was 1.11 and 1.21 N/g at harvest (Fig. 6 and Table 1). In general, fruit characteristics and ripening patterns were similar to those reported by Di Vaio *et al*. (2013) for the same cultivars.

Despite the high canopy volume of the trees, the mechanical harvesting yield (percentage of mechanically harvested olives) was very high: 96.58% and 96.74% for the 'Ortice' and 'Ortolana' cultivars, respectively (Table 1). These high values are likely due to the relatively low olive detachment/weight ratios at the time of harvesting, which were close to one. Indeed, Farinelli *et al*. (2012 a) reported a significant negative relationship between the olive detachment/weight ratio and the mechanical harvesting yield obtained with a trunk shaker. The same authors observed that high mechanical harvesting yields are obtained when the ratio is less than 2. In this regard, it can be noted that in the present work, for both cultivars, the olive detachment/weight ratio, as a result of the medium weight and the medium/low detachment force of the olives, was less than 2 for the entire ripening period. This indicates that mechanical harvesting could be carried out efficiently

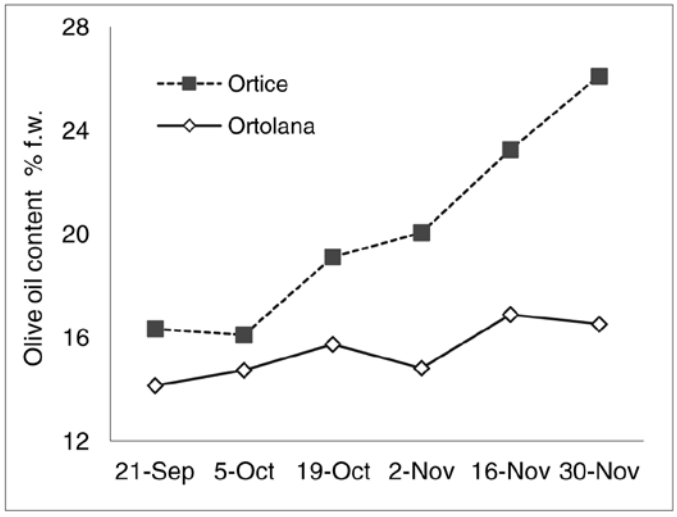

Fig. 4 - Evolution of olive oil content (% f.w.) during maturation.

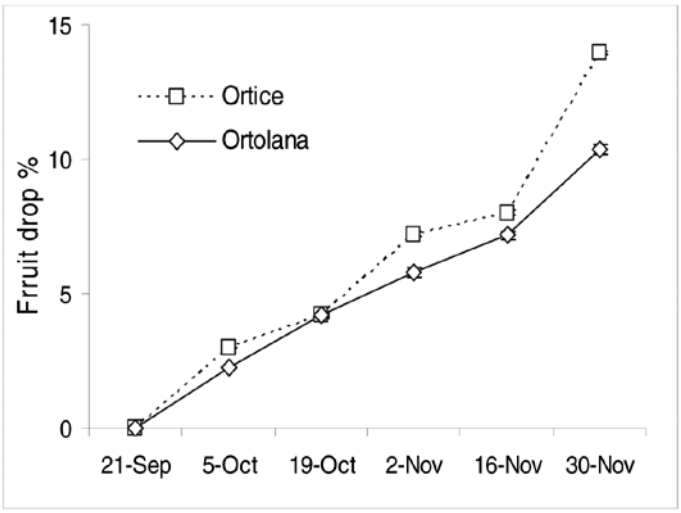

Fig. 5 - Evolution of pre-harvest fruit drop during maturation.

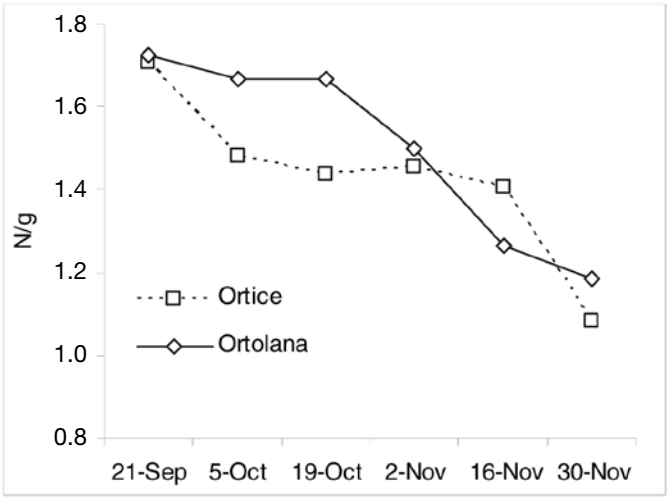

Fig. 6 - Evolution of the ratio between olive detachment force and drupe weight during maturation (mean ± standard error).

Table 2 - Quality indices of oils obtained from cv. Ortice and Ortolana olives mechanically harvested with a trunk shaker (mean±standard error)

Cultivar	Acidity % oleic acid	Peroxide value meq O_2 Kg^{-1}	UV			Defects at panel 0-5
			K232	K270	Δk	
Ortice	0.52±0.04	8.2 b±0.13	1.967±0.065	0.145±0.014	-0.003±0.000	0±0.00
Ortolana	0.46±0.02	6.5 b±0.14	1.824±0.032	0.155±0.036	-0.005±0.000	0±0.00

during the entire ripening period. At the farm level, this allows for flexibility, also considering that oil quality changes during olive ripening and so oils with different characteristics can be obtained by modulating harvesting time (Inglese *et al*., 2011). Moreover, the low labour requirement (only two workers), the reduced time for operation (about 2.50 min/tree, which included the approach to the tree and attachment of the shaker to the trunk, opening of the reversed umbrella, shaking and closing the reversed umbrella), the good yield/tree and high quantity of harvested fruit allowed very high values of work productivity to be obtained: about 302.04 kg/h/worker for 'Ortice' and 245.88 kg/h/worker for 'Ortolana' (Table 1). This result highlights the importance of a good yield/tree in determining high work productivity and therefore the economic convenience of using machines for olive harvesting (Famiani *et al*., 1998). The oil quality indices (acidity, peroxide number, spectrophotometric indices and sensorial characteristics by panel test) reported in Table 2 show that all analytical and sensory evaluations of the oils from the two cultivars met the requirements set by law for extra virgin olive oils (EC Regulation n. 2568, 1991). Therefore, the use of the trunk shaker with interceptor allowed high quality oils to be obtained.

In conclusion, the results of the present study show that the trunk shaker with the reversed umbrella interceptor, which allows for high harvesting yields and high labour productivity, can be considered an interesting solution for mechanical harvesting of the autochthonous cultivars 'Ortice' and 'Ortolana' in southern Italy.

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Factors influencing mating incidence and reproduction in codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae)

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Key words: Cydia pomonella, fecundity, fertility, mating ability, mating frequency.

Abstract: **The codling moth *Cydia pomonella* L. is a primary pest of apple and various studies have been performed to assess the possibility of applying sterile insect technique as a control method against this pest. In support of this technique, the present work aims to examine the effects of adult age and weight on mating ability, number of matings, fecundity and fertility in *C. pomonella*. The relationship between number of matings, fecundity, and fertility of females was also studied. Female and male weights were found to have an effect on the number of times individuals mate, but male weight only influenced mating success. Unlike male weight, female weight affected fecundity and fertility. Negative correlations were found between mating success, fecundity and fertility and adult age. Multiply-mated females and those which did not accept a second mating showed higher fecundity and fertility than their counterparts that were not given the opportunity to remate. Our results provide essential information necessary to increase the effectiveness of sterile insect technique as a control method against *C. pomonella*.**

1. Introduction

Apple is a very important fruit tree in Syria with total apple acreage of about 41 000 ha. Codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) is considered the most important insect pest of apple in Syria and insecticides are widely used to control it. Such control methods are costly, nonselective, environmentally unsafe and effective for only a short period in the treated area. Moreover, *C. pomonella* has already developed resistance to various insecticides (Varela *et al*., 1993). Therefore, a more reliable and environmentally safe control method is required.

Knipling (1970) and Myers *et al*. (1998) reported that the sterile insect technique (SIT) can be considered an important component of an area-wide approach to insect control programs. The possibility of applying SIT as an alternative control method to suppress *C. pomonella* populations has been determined by many researchers (Bloem *et al*., 1999, 2001). This approach relies on mass-rearing and release of both sexes of irradiated moths into wild pest populations.

The success of this technique against codling moth depends largely on the release of sexually competitive insects capable of locating and carrying out mating with several feral individuals (Knipling, 1981). It is widely agreed that insects with a long lifespan, good dispersal pattern, high incidence of mating, ability to transfer sperm successfully, and a noticeable fecundity would exhibit an acceptable level of mating competitiveness (Carpentar *et al.,* 1989).

Mating in Lepidoptera involves the following sequence of events: copulation, spermatophore transfer, insemination and egg fertilization. Bues *et al*. (1992) observed that most lepidopteran females tend to mate within 24 h of emergence. The effect of adult age on mating varies considerably between species as well as sex. Previous studies have stated that younger females of *Pectinophora gossypiella* Saunders (Lingren *et al.,* 1988), *Eoreuma loftini* Dyar (Spurgeon *et al*., 1995), *Lymantria dispar* L. (Proshold, 1996), and *Phthorimaea operculella* Zeller (Makee and Saour, 2001) had a higher occurrence of successful matings compared to older individuals. On the contrary, young females of *Ephestia kuehniella* Zeller showed a low mating incidence. However, the newly emerged males of *E. kuehniella* were more likely to transfer spermatophores than older males (Calvert and Corbet, 1973). The effect of adult age on reproductive capacity is well known in several lepidopteran species (Ellis and Steele, 1982). It has been shown that the reproduction of *Plutella xylostella* (L.) females was significantly reduced by age (Nemoto *et al*., 1992).

Insect weight plays an important role in mating and reproduction. Large males of *Ephestia elutella* Hübner were more likely to mate than smaller ones (Phelan and Barker 1986). Similarly, a significant relationship between body size and successful mating has also been reported in the dipteran *Drosophila melanogaster* Meigen (Partridge and Farquhor, 1983). Studies have shown that in hemipteran *Podisus macu-*

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liventris and *P. connexivus* the heavier females presented a better reproductive rate (Evans, 1982; Zanuncio *et al*., 1992).

To maximize the mating competitiveness of released insects, the influence of adult age and weight on mating incidence and reproduction capacity of codling moth were examined. In addition, the effect of the number of matings on fecundity and fertility of this species was tested.

2. Materials and Methods

A *C. pomonella* colony has been maintained in our laboratory for several years and was used for the present study. Newly emerged adults were crossed in Petri dishes (12 cm dim), five pairs in each. A wet cotton wool was placed in each Petri dish as drinking source. After 3-4 days, the dishes which had eggs were collected and soaked with 2% Sodium hypochlorite solution for 2 min for egg sterilization. Then the dishes were washed with tap water and left to dry. Eggs were checked daily for hatching. Newly hatched larvae were placed on artificial media consisted of the following ingredients: agar-agar, maize, wheat germ, casein, yeast, Wesson salts, benzoic acid, fumidil, ascorbic acid, vitamins and nipagine (Anisimov, personal communication). All insect stages were kept under constant temperature at 25±1°C with 70±5% RH, and a photoperiod of 16:8 h (L:D).

In all the experiments, pupae were sexed and individually placed in small plastic tubes until eclosion. For oviposition, newly emerged females and males were paired in Petri dishes (12 cm dim) having a feeding source (a wet cotton wool). "Mating incidence" in this study is used to indicate successful spermatophore transfer and/or spermatophore presence in the bursa copulatrix of the female. The number of matings is reflected by the number of spermatophores in the bursa copulatrix.

The effect of adult age

Ten different groups of virgin females (n= 20 in each group) aged 1-10 d were employed. Females of each group were individually paired with newly emerged males (<18 h). After 24 h, males were removed and females were kept for oviposition until death. All eggs were collected, counted and allowed to hatch. After death, the females were dissected and examined for the presence of spermatophores in the bursa copulatrix.

Ten different groups of virgin males (n=20 in each group) aged 1-10 d were examined to determine the effect of male age on successful spermatophore transfer. Males of each group were individually paired with newly emerged virgin females (<18 h). After 24 h, males were removed and females were kept for oviposition until death. All eggs were collected, counted and allowed to hatch. After death, the females were dissected and examined for the presence of spermatophores.

The effect of adult weight

To determine the influence of male and female weight on incidence of mating, number of matings, fecundity and fertility pupae were weighed, sexed, and divided into three groups based on weight: light, medium and heavy. Female pupal weights were, respectively, 34-37, 39-42 and 44-47 mg, while male pupal weights were, respectively, 25-31, 36-45 and 47-49 mg. Emerged females and males in each group were individually paired with newly emerged adults of the opposite sex. In male and female groups, males were paired with females weighing 36.2±0.5 mg, while females were paired with males weighing 30.6±1.3 mg.

In each group, the females and males were kept together until death. All eggs were collected, counted and left to hatch. The females were dissected and the number of spermatophores in the bursa copulatrix were counted. To determine the relationship between adult weight and fecundity and fertility, only mated females were considered.

Effect of sex ratio on mating ability

Two experiments were carried out. In the first experiment, 1-d-old males (n = 20) were individually confined with three newly emerged females (1 male: 3 females). Males and females were kept together for 24 h, after which time the females were dissected and the number of spermatophores was determined.

In the second experiment, 1-d-old females (n= 20) were singly confined with three newly emerged males (1 female: 3 males). After 24 h, females were removed, dissected and the number of spermatophores was determined. A ratio of 1 female: 1 male was used as a control group for the two experiments.

Effect of female multiple mating on fecundity and fertility

Two groups of virgin females were used. Females of the first group (n= 20) were individually paired with newly emerged males. After 24 h, males were removed and females were kept for oviposition until death. In the second group, females (n= 45) were individually paired with 1-d-old males. Males were removed after 24 h and replaced with new 1-d-old males. The same procedure was followed for seven successive days. In both groups, all eggs were collected, counted and allowed to hatch. After death, the females were dissected to determine the presence and number of spermatophores.

Statistical analysis was carried out using the STATISTIC program version 6 (Statsoft, Inc. 2003) at 5% level (P= 0.05). A simple linear regression analysis was done to study the relationship between adult age and incidence of mating and fertility. Data were subjected to analysis of variance for determination of differences between means, which were tested for significance using Tukey HSD test. The percentages were analyzed by applying normal approximation test (analysis of proportion).

3. Results

Effect of adult age

Figure 1 illustrates the effect of male and female mating ability of *C. pomonella* with regard to age. A regres-

sion line was fitted to present the relationship between incidence of mating and adult age. The percentage of mating ability was significantly correlated with adult age (y= -7.2303x + 95.667, R^2 = 0.77, P<0.05; y = -7.8788x + 90.133 R^2 = 0.86, P<0.05 for females and males, respectively). A significant increase in mating ability was recorded when males and females became 2 d old. After that, a significant reduction in the mating ability was noticed 4 d and 5 d after male and female emergence (Fig. 1). The mating ability of 1-d-old males and females was similar, and afterwards the mating ability of females was higher than that of males, regardless of adult age.

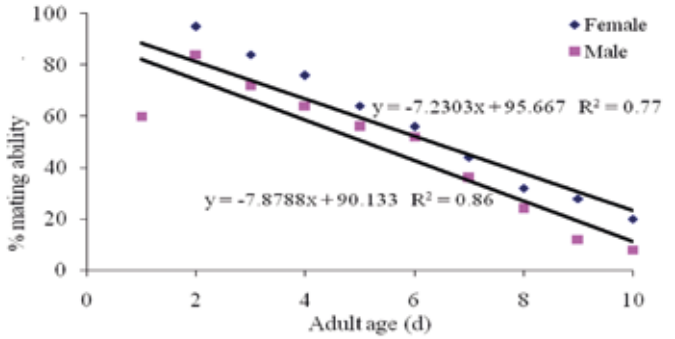

Fig. 1 - Effect of adult age on mating ability of codling moth.

To determine the effect of adult age on fecundity and fertility only mated females were used in the analysis. Figure 2 reveals that the number of eggs increased when the adults became 2-d-old; the number of eggs then significantly declined (F=13.19; d.f=9,190; P<0.05 for female and F=12.9; doff=9,190; P<0.05 for male). Significant differences were noticed between males and females at each tested age, except when both sexes were 1-d-old (Fig. 2).

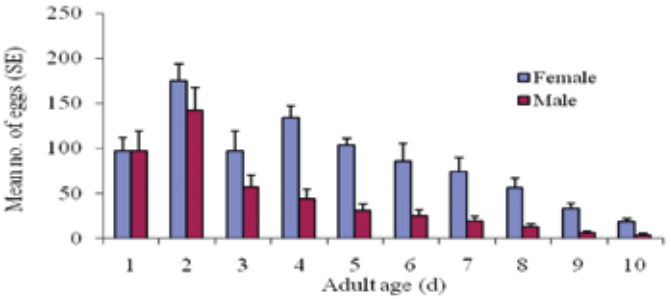

Fig. 2 - Effect of adult age on mean fecundity of codling moth female.

Figure 3 illustrates that as females and males got older their fertility decreased. There was a strong relationship between adult age and fertility (y= -5.3455x + 87.8 R^2 = 0.90 and y= -8.5939x + 91.867 R^2= 0.93 for females and males, respectively). Regardless of adult age, female fertility was significantly higher than that of males except at age 1 d.

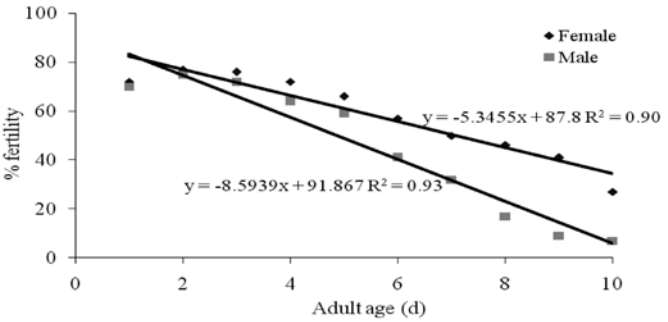

Fig. 3 - Effect of adult age on adult fertility of codling moth.

Effect of body weight

The percentage of mating ability of the females was not affected by their weight. No differences in mating ability were found among the three tested female weight groups (Fig. 4). In contrast, the number of female matings was influenced by their weight. The mean number of matings of group 3 females (the heaviest) was significantly higher than that of groups 1 and 2 (F=8.6; d.f=2,74; P<0.05). However, the mean number of matings of group 2 females did not significantly differ from that of group 1 females (Fig. 5).

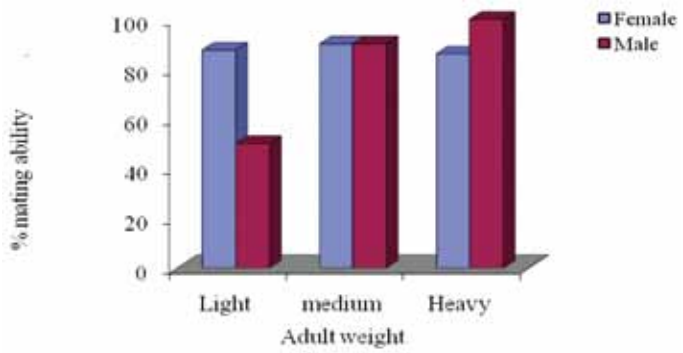

Fig. 4 - Effect of body weight on mating ability of codling moth adults.

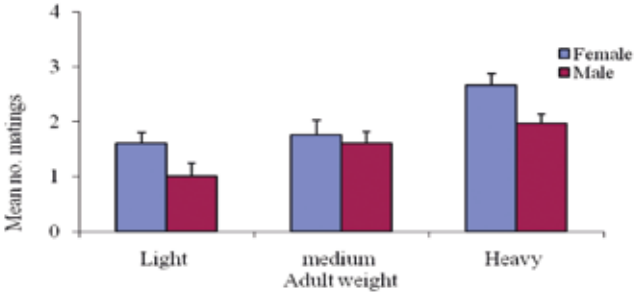

Fig. 5 - Effect of body weight on number of mating of codling moth adults.

A significant difference in the percentage of mating incidence was observed among male weight groups 1 and 3 (Fig. 4). All males of group 3 (the heaviest) were able to transfer spermatophores, whereas only half of group 1 males (the lightest) was able to do so. The mating incidence of group 2 males did not significantly differ from that of group 3 (Fig. 4). The mean number of matings of group 1 males was significantly lower than that of groups 2 and 3 (F=5.19; d.f=2,72; P<0.05). No differences in the number of matings were detected between males in groups 2 and 3 (Fig. 5).

Results from the study show that female body weight significantly affects fecundity (F=6.24; d.f=2,74; P<0.05) and fertility (F=7.09; d.f=2,74; P<0.05) of the codling moth. The mean number of eggs laid and mean number of eggs hatch increased significantly when female weight increased (Table 1).

Table 1 - Effect of female body weight on fecundity and fertility of *C. pomonella*

Female weight (mg)	Mean fecundity	Mean fertility
34-37	66.24±11.35 b	50.6±9.37 b
39-42	99.80±12.70 ab	68.9±11.74 b
44-47	134.11±16.03 a	112.5±14.14 a

Means followed by different letters (columns) are significantly different at P<0.05 (Tukey HSD test).

Unlike female weight, male weight did not affect fecundity and fertility. There were no significant differences in fecundity and fertility between heavy and light males (Table 2).

Table 2 - Effect of male body weight on fecundity and fertility of *C. pomonella*

Male weight (mg)	Mean fecundity	fertility
25-31	63.16±12.85 a	52.36±10.8 a
36-45	85.04±9.88 a	57.80±8.6 a
47-49	95.32±12.2a	67.04±9.8 a

Means followed by different letters (columns) are significantly different at P<0.05 (Tukey HSD test).

Effect of sex ratio on mating ability

When *C. pomonella* females were confined with one or three males for 24 h, they mated only once. The incidence of female mating did not differ significantly when confined with one or three males (Table 3). *C. pomonella* males mated only once when paired with a single virgin female for 24 h. When a male was confined with three females, it mated more than once (Table 3).

Effect of female multiple mating on fecundity and fertility

When *C. pomonella* females were exposed to newly emerged males for seven successive days (group 2 females), 18% of them mated once, more than half mated twice or three times and 18% mated four or five times (Table 4).

Table 3 - Effect of number of females and males on number of mating of C. *pomonella* during a 24-h period

Sex	Sex Ratio F:M	% mated adult per no. of mating		
		0	1	2
Female	1: 1	B35 ab	A65 a	0
	1: 3	B40 a	A60 a	0
Male	1: 1	B30 ab	A70 a	0
	3: 1	B20 b	A60 a	B20

Percentages preceded by different capital letters (raws) and followed by different small letters (columns) are significantly different at P< 0.05 (normal approximation test).

Table 4 - Effects of repeated mating on the mean number of eggs and egg hatch percentage of *C. pomonella* females

Female group	No. of matings	Females (%)	Mean no. of eggs/female±SE	Mean fertility/female± SE
1	1	60	93.6±19.9 b	93.6±19.9 b
2	0	7	134.7±25.6 b	0
	1	18	237.1±32.1 a	237.1±32.1 a
	2	35	225.8±20.5 a	225.8±20.5 a
	3	22	260.9±27.9 a	260.9±27.9 a
	4	11	238.6±8.5 a	238.6±8.5 a
	5	7	270.0±25.8 a	270.0±25.8 a

Means and percentages followed by different letters (columns) are significantly different at P<0.05 (Tukey HSD test).
Group 1= females were paired with the males only for 24 h (n = 20).
Group 2= females were paired with new virgin male every 24 h for 7 successive days (n = 45).

The mean number of eggs and fertility of group 1 females, in which the females were not given an opportunity to remate, were significantly lower than those of group 2 females, in which the females were given a chance to remate (F=6.7; d.f=6,59; P<0.05 for fecundity, F=6.7; d.f=6,59; P<0.05, respectively). Regardless of the number of matings of group 2 females, the mean number of eggs and fertility did not differ significantly.

4. Discussion and Conclusions

The tendency and number of matings of *C. pomonella* may be affected by various factors. Our results indicate that old males and females of this species were less likely to mate than young individuals. However, differences appeared when the patterns of mating ability of females and males were compared. The female mating ability was greater than that of males. However, the mating ability of females declined less rapidly than that of males with age (Fig. 1). In Lepidoptera, the ability to release adequate sex pheromone and/or to respond to the sex pheromone of the

opposite sex generally reduces with age (Spurgeon *et al*., 1995; Proshold, 1996; Makee and Saour, 2001). Our result confirms that senescence might influence male moths to a greater degree than females.

In the current study, male age greatly influenced female fecundity and fertility of *C. pomonella* (Figs. 1 and 2). Conflicting results have been reported on the impact of male age on female fecundity and fertility of *C. pomonella.* Vickers (1997) reported that male age had no effect on female fecundity and fertility, whereas Knight (2007) showed that female fecundity after mating with 1-d-old males was significantly lower than after mating with 3-d-old males. Similarly, female fecundity and fertility of *Plutella xylostella* L. decreased when the females mated with old males (Nemoto *et al.,* 1992; Wang *et al.,* 2011). Female discrimination against older males has been demonstrated in several species (Ritchie *et al.,* 1995; Jones and Elgar, 2004). Thus, *C. pomonella* females preferred mating with younger males since mating with older males diminishes female fecundity and fertility. Such reduction in reproductively could be due to an age-correlated reduction in sperm quality (Crow, 1997; Hansen and Price, 1999).

The results from the present work clearly indicate both fecundity and fertility were affected by female age at mating, both of which decreased with an increase in age (Figs. 1 and 2). Similar effects have been observed previously in several species such as *Spodoptera exigua* (Hübner), (Rogers and Marti, 1996), *Lobesia botrana* (Dennis & Schiffermüller) (Torres-Vila *et al*., 2002) and *P. xylostella* (Wang *et al*., 2011). The reduction of egg production and viability when mating of *C. pomenella* was delayed after emergence could be related to utilization of the fat body, which is essential source of vitellogenins and lipids for oocyte maturation, for non reproduction metabolism by older females (Barrer, 1976).

Unlike male weight, female weight did not play a role in mating ability in *C. pomonella* (Fig. 4); both light and heavy females had similar mating tendencies. However, female weight did affect the number of matings (Fig. 5). Similar results were reported in *P. operculella* (Makee and Saour, 2001). Male weight had important impact on mating ability and number of matings of *C. pomonella* (Figs. 4 and 5). There may be two main reasons for the relationship between male weight, ability to produce and transfer spermatophore, and number of matings: (1) heavy males may be able to produce sufficient quantity of sex pheromone to attract females (Thornhill and Alcock, 1983; Phelan and Barker, 1986); (2) *C. pomonella* females tend to mate with heavier males, as confirmed by our data (i.e. they mated with 100 and 50% of heavy and light males, respectively) (Fig. 4).

Our results show that reproductively of heavy females was greater than for light females (Table 1). Strong correlation between adult weight and fecundity has been noted in various insect species (Evans, 1982). Honek (1993) reported that genetic and environmental factors could influence insect weight. There are several environmental factors including food type and temperature (Mohaghegh *et al*., 1999). Generally, female weight partly reflects the size of fat-body. This organ is essential for oocyte maturation since it is a site of lipid and yolk protein synthesis (Chapman, 1982). Therefore, heavy females are able to produce more eggs since they have a larger fat-body. In contrast to female weight, male weight did not influence the fecundity and fertility of *C. pomonella* (Tables 1 and 2).

Like most lepidopteran, when a *C. pomonella* male was paired with one female for 24 h, it was able to produce and transfer only one spermatophore (Makee and Saour, 2001). Nevertheless, if several virgin females were available, *C. pomonella* males were able to transfer more than one spermatophore during one scotophase (Table 3). A comparable result has been noted in males of *Grapholitha molesta* Busck, *Spodoptera frugiperda* (J.E. Smith) and *P. operculella* (George and Howard, 1968; Simmons and Marti, 1992; Makee and Saour, 2001).

Nevertheless, *C. pomonella* females mated once in 24 h even when they were confined with three newly emerged males (Table 3). Thus, females needed a lapse of time to remate, regardless of the number of males available. A similar result was reported in *P. operculella* (Makee and Saour, 2001). After mating, females released special volatile materials that reduced their receptivity (Tompkins and Hall, 1981).

When *C. pomonella* females were exposed to newly emerged males for seven successive days, only 18% of them mated once, 35% twice and 40% several times (Table 4). *C. pomonella* females were capable of mating more than five times when they were paired with virgin males for seven successive days. Conversely, *P. operculella* females were unable to mate more than three times when they were paired with virgin males for seven successive days (Makee and Saour, 2001). Whatever the number of matings, the mean number of eggs and fertility of females were similar in both species. This may imply that once-mated females would not seek additional matings since they received sufficient effective sperm during their first mating. Several studies reported that *P. operculella*, *Heliothis virescens* F. and *L. dispar* females that did not receive an adequate quantity and quality of sperm during the first mating needed to remate (Lingren *et al*., 1988; Proshold, 1995; Makee and Saour, 2001). On the contrary, Knight (2007) stated that *C. pomonella* females that had mated three times had a significantly higher fecundity than singly-mated moths.

Fecundity and fertility of females with an opportunity to remate were higher than those of once-mated females that were not allowed to remate (Table 4). The relationship between the number of matings and the female's reproductivity could be attributed to: (1) sperm replenishment which is required for egg fertilization; and (2) nutrients derived from spermatophores that are utilized by the female in egg production (Greenfield, 1983).

The present study provides useful information for situations where sterile insect technique could be considered against *C. pomonella*: (1) repeated releases of young sterile insects should be executed rather than one major release; (2) production of heavy insects is preferable in mass-rearing procedures; (3) the effectiveness of sterile in-

sect technique against *C. pomonella* would not restrain by the release of both sterile males and females, since during one scotophase *C. pomonella* males could remate whereas females were unable to do that.

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Discrimination of grapevine varieties cultivated in the Czech Republic by Artificial Neural Networks

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Key words: ampelography, phyllometry, *Vitis vinifera*, variety identification.

Abstract: An artificial neural network approach, based on fractal leaf parameters, and classical ampelography were used to identify nine grapevine varieties cultivated at the St. Claire's vineyard, Prague Botanic Garden. Fifty healthy, fully-expanded leaves were collected for each variety, scanned using an optical scanner and then elaborated by computer programs. Fourteen phyllometric parameters were qualitatively and quantitatively analysed by the digital image analysis. Comparative frames were constructed for each variety and the relationships among varieties were assessed using artificial neural networks. Results were then compared with the outcome from traditional ampelographic analysis. The Artificial Neural Network technique appears to be a complementary approach to the traditional ampelography methods commonly used for cultivar discrimination, since the equipment necessary for this analysis is very inexpensive and available. Application of the technique led to the distinction of nine selected varieties of *Vitis vinifera*.

1. Introduction

Ampelography is a traditional morphological method used for the identification and discrimination of varieties of grapevine (*Vitis vinifera* L.). It has also been found to be very useful in detecting and describing variability among chosen potential clone candidates (Poljuha *et al*., 2006). Nowadays, a link between historic descriptions and the molecular fingerprints is also very important, especially in cases of possible homonymy and synonymy of autochthonous varieties (Cervera *et al*., 2001). Official descriptors have been published to be used together with the analysis of germplasm material (Dettweiler, 1993; Ortiz *et al*., 2004).

All the organs used in ampelography (leaves, grapes, shoots etc.) usually change their aspect during phenologic phases, hence it is important to find the characteristics able to discriminate between varieties according to these phases: shoots in the early stage, leaves in the moment when they are mature, and later also the grapes and mature berries (Cancellier, 2007).

The use of fractal-based measurements of digitally-acquired images eliminates problems with different phenologic phases as well as problems with subjectivity. This allows defining the good shape measure that can be effectively applied to leaf shapes, so they can be compared and analysed by meaningful and objective criteria (Mancuso, 1999). Using fractal parameters and phyllometric outputs, an artificial neural network can be constructed and effectively used to differentiate varieties and accessions. It is an easy method that requires low-cost equipment such as an optical scanner, personal computer and free software (Mugnai *et al*., 2008).

St. Claire's Vineyard is an historical area located close to the centre of Prague, and where a long tradition of grapevine cultivation dates back to the 13th century. Following a period of decline, the vineyard is today regaining its former importance. New varieties are being planted and no ampelographic observations have yet been carried out.

The main aim of the present work was to discriminate the grapevine varieties cultivated under the climatic conditions of Prague (Czech Republic) using either the subjective method according to the international descriptors published by IPGRI (1997), or an objective computing method which consists of analysis using an artificial neural network. These two approaches are also compared in this paper to assess differences between subjective and objective analysis.

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2. Materials and Methods

Plant material

Leaf samples for the analysis of nine varieties were collected at the St. Claire's Vineyard, Prague Botanic Garden. Nine varieties (Table 1), cultivated in a sufficient quantity to provide a sufficient source of samples, were chosen for analysis. Berry and grape samples were collected at the time of the harvest, which was done according to the further utilization of the grapes.

Table 1 - List of selected varieties

	Variety
1	'Müller Thurgau'
2	'Gewürztraminer'
3	'Rhine Riesling'
4	'Italian Riesling'
5	'Moravian Muscat'
6	'Sauvignon'
7	'Blue Portugal'
8	'White Chasselas'
9	'Red Chasselas'

Morphological and phenological characterization

For the classical ampelography, the following method was applied. In the period 2006-2008, 22 morphological and six phenological traits were evaluated in 10 randomly chosen plants for each variety, according to the Descriptors for Grapevine (IPRGI, 1997). All parameters chosen for the study are listed in Table 2 with the codes of updated descriptors (OIV, 2009).

Digital image analysis of leaves, phyllometric parameters and fractal analysis

For each variety, 50 healthy, fully-expanded leaves were collected from ten randomly selected plants in late spring 2008. Leaf images were acquired (200 dpi, 256 greyscale) using an optical scanner. Based on the method described by Mancuso and Nicese (1999), 14 phyllometric parameters (Table 3) were determined for each leaf using image-analysis software (UTHSCSA Image Tool Program 3.0).

Table 2 - List of characteristics selected for the description of observed cultivars. The codes are in line with OIV guidelines (OIV, 2009)

Character code	Description
001	Young shoot: aperture of tip
003	Young shoot: intensity of anthocyanin colouration on prostrate hairs of tip
006	Shoot: attitude
007	Shoot: colour of dorsal side of internode
008	Shoot: colour of ventral side of internode
051	Young leaf: colour of the upper side of blade (4th leaf)
065	Mature leaf: size of blade
067	Mature leaf: shape of blade
068	Mature leaf: number of lobes
070	Mature leaf: area of anthocyanin colouration of main veins on the upper side of blade
076	Mature leaf: shape of teeth
079	Mature leaf: degree of opening/ overlapping of petiole sinus
102	Woody shoot: structure of surface
103	Woody shoot: main colour
202	Bunch: length (peduncle excluded)
204	Bunch: density
220	Berry: length
223	Berry: shape
241	Berry: formation of seeds
225	Berry: colour of skin
236	Berry: particularity of flavour
303	Time of beginning of berry ripening (véraison)
233	Berry: must yield
502	Bunch: weight of a single bunch
503	Single berry weight
505	Sugar content of must

The codes in bold letters indicate the characteristics which were evaluated by ANOVA.

Table 3 - Fourteen phyllometric parameters measured by image analysis software

	Parameter	Definition
1	Area	The area of the leaf
2	Perimeter	The perimeter of the leaf
3	Major axis length	The length of the longest line that can be drawn through the leaf
4	Minor axis length	The length of the longest line that can be drawn through the leaf perpendicular to the major axis
5	Roundness	Computed as: $(4*\pi*\text{area}) / \text{perimeter}^2$
6	Elongation	The ratio of the length of the major axis to the length of the minor axis
7	Feret diameter	The diameter of a circle having the same area as the leaf
8	Compactness	Computed as: $\text{sqrt}(4*\text{area}/\pi) /$ major axis length
9	Integrated density	Computed as the product of the mean grey level and the number of pixels in the image of the leaf
10	Minimum grey level	Minimum grey level of the leaf
11	Mean grey level	Mean grey level of the leaf
12	Median grey level	Median grey level of the leaf
13	Mode grey level	Mode grey level of the leaf
14	Maximum grey level	Maximum grey level of the leaf

The fractal spectrum of the leaves was obtained using fractal image analysis software (HarFA, Harmonic and Fractal Image Analyzer 4.9.1,), according to the method described by Mancuso (2002). Briefly, greyscale image of each leaf was thresholded for a grey value between 0 and 255 and the fractal dimension for each grey value was then assessed using the box counting method. The implementation of these methods has been described in detail by Mancuso *et al*. (1999). After drawing the baseline (fractal dimension = 1) which separates the fractal (>1) from the non-fractal (<1) zone of the spectrum, five fractal parameters (First X, Peak X, Last X, Peak Y and Total Peak Area) were calculated (Fig.1 A).

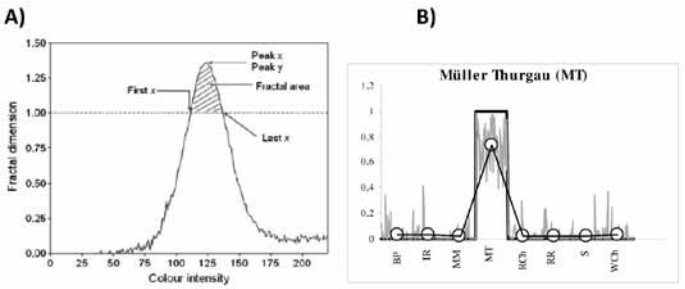

Fig. 1 - Fractal spectrum of one leaf and identification of five parameters to be implemented in the ANN. Graphical representation of Fractal parameters: Theoretical situation (A); and a real value (B).

Artificial Neural Network analysis

An Artificial Neural Network (ANN) was constructed as previously described in Pandolfi *et al*. (2009 a). Fourteen phyllometric parameters and five fractal parameters were used as input layers, and the nine grapevine accessions represented the output. To optimize the neural network activity, the number of hidden neurons and the number of iterations was modified. With regard to the hidden layer, many factors (such as learning scheme, numbers of nodes of the output and input and connections between them) play an important role for the determination of the best configuration (Zurada and Malinowsji, 1994). The ANN outputs are represented by a XY-graph for each accession, with the accession names on the x-axis, and the y-axis representing the output. Each graph aims to show how the ANN was able to discriminate the selected accession in comparison with the others. The level of similarity is expressed by number, which ranges between 0 (false) and 1 (true) (Fig. 1 B). Due to the natural variability among leaves, the output of the expected class tends to report a value close to 1, but less than 1, while the others should be close to 0 (Mugnai *et al*., 2008).

Statistical analysis

Statistical analysis of the selected characteristics was performed by analysis of variance (ANOVA) and a comparision was done using the Turkey HSD Test (program Statistica 7.0 CZ).

The graphical presentation of variability of the tested varieties was carried out using Principal Component Analysis (PCA) (software Statistica 7.0 CZ).

NTSYS 2.1 was used to investigate neural network outputs performing a cluster analysis by Unweighted Pair Group Method Analysis (UPGMA) based on the similarity matrix calculated using the cosine function (Eq. 1).

Equation 1:

$$COSINE_{(x,y)} = \frac{\sum_i (x_i y_i)}{\sqrt{\left(\sum_i x_i^2\right) x \left(\sum_i y_i^2\right)}}$$

3. Results

Morphological data

Seven characteristics (Table 2), as evaluated by ANOVA, showed differences between the varieties during the three consecutive years of sampling, as well as variability within the variety. The number of lobes observed in the mature leaves varied during the study period in three varieties ('Müller Thurgau', 'Rhine Riesling', and 'Blue Portugal').

A significant variation was observed both in the weigh of a bunch and the weight of berries, as well as in the size of berries within one variety.

With regard to must yield and its sugar content, there was no significant variance among the varieties, however all the varieties demonstrated a significant correlation between the sugar content of the must and the time of berry ripening (r=0.74). The highest sugar content was measured in 2006, when the mean temperatures remained quite high during whole vegetative period (in July as high as 25°C), whilst the total precipitation was about 5 mm in July.

For most of the 19 characteristics that were not included in the ANOVA analysis, there was considerable stability over the course of the years. The greatest variability was shown in the size of a blade of a mature leaf; it remained stable only in 'Rhine Riesling'. In the other eight varieties, the size of the leaves varied significantly.

Correlations between morphological and phenological characters were also assessed. The output obtained from both morphologic and phenological characteristics after three years of observations (Fig. 2) was a score plot cre-

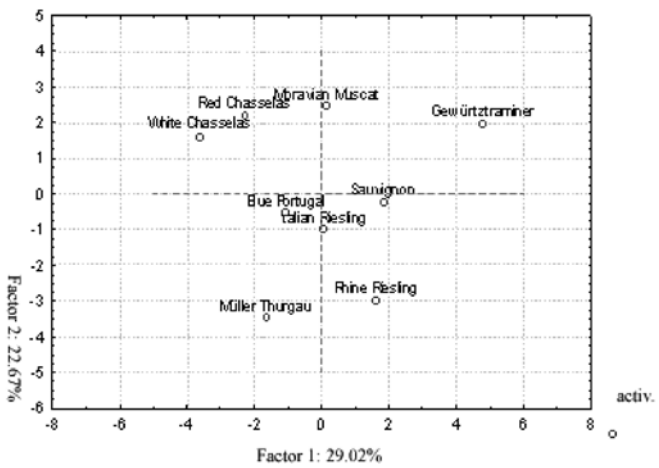

Fig. 2 - PCA representation.

ated by PCA analysis. According to the graph, factor 1 explained 29.02% of the total variation, whereas factor 2 expressed 22.67% of the total. Evaluating both approaches (morphological and phenological), 'Red Chasselas' and 'White Chasselas' were identified as being quite close, which indicated their relationship to one another. The second group was formed in the very centre of the graph and includes 'Blue Portugal', 'Italian Riesling' and 'Sauvignon'. More distant from the centre group and also from each other were 'Müller Thurgau' and 'Rhine Riesling'. The only variety which remained completely separate was 'Gewürztraminer'.

Neural network analysis

In all eight cases, the artificial neural network was able to recognise all the accessions presented in the learning phase. Throughout there was a clearly defined peak for the selected accession; therefore the varieties were well-separated one from another. The highest level of similarity was present in 'Müller Thurgau' (0.810). However, all other accessions showed quite high average output in a range from 0.541 ('Rhine Riesling') to 0.657 ('Moravian Muscat') (Fig. 3).

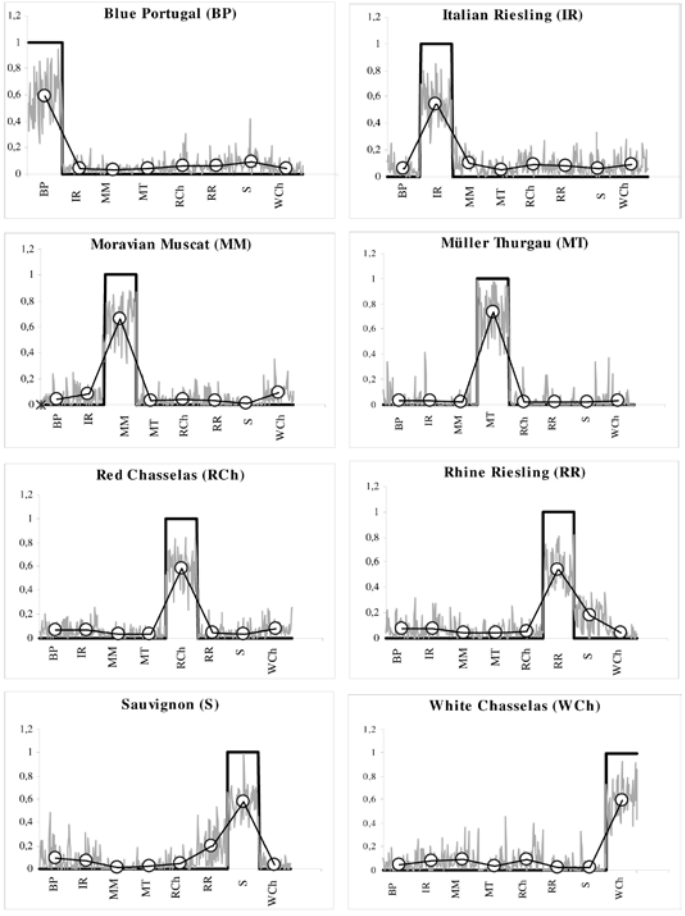

Fig. 3 - Neural networks of eight chosen varieties.

'Müller Thurgau' also showed the lowest degree of similarity with the other varieties and this was confirmed by the UPGMA diagram, where 'Müller Thurgau' remained completely separated from all other varieties.

Another interesting result was the position of both 'Chasselas' in the neural network diagram and the final dendrogram. In the dendrogram, 'Red Chasselas' was more closely related to 'Italian Riesling' than to 'White Chasselas'.

Using the data from the neural network, Euclidean distances were calculated and a dendrogram based on the distance matrix data, by applying an Unweighted Pair Group Method with Average Mean (UPGMA) cluster analysis, was constructed (Fig. 4).

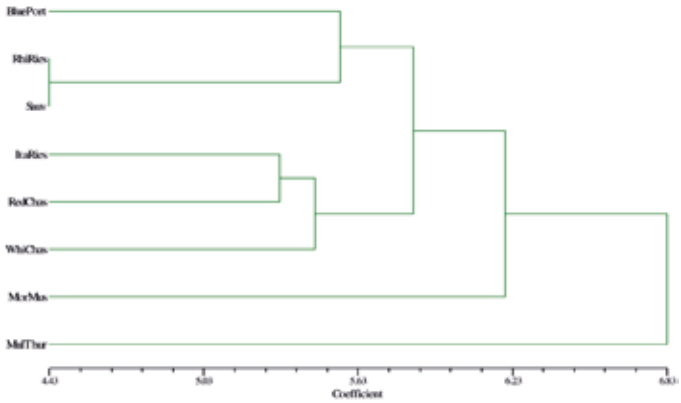

Fig. 4 - UPGMA dendrogram.

The dendrogram formed two clusters, and left two varieties more ('Müller Thurgau') or less ('Moravian Muscat') separated from the others. Two other varieties, 'Rhine Riesling' and 'Sauvignon', were indicated at the same level. Even though they were the two most related samples, they still remained quite distant, with the similarity coefficient at 4.43. The results showed a closer relationship between 'Italian Riesling' and 'Red Chasselas' than the relationship between both 'Chasselas', even though they are considered to be colour variations of the same variety.

4. Discussion and Conclusions

In the present investigation all general characteristics which are common to *Vitis vinifera*, without regarding the differences between the varieties (IPGRI, 1997), were confirmed with regard to morphology for the varieties under study.

There was only a slight variation in the number of lobes observed in the mature leaves, which is due to the genotype fixation of this trait, as proposed by Poljuha *et al.* (2006). In three varieties ('Müller Thurgau', 'Rhine Riesling' and 'Blue Portugal') the number of lobes varied during the observation period. This way have occurred due to the unisindentification of the depth of the status. (Cancellier, 2007).

The size of a berry was strongly correlated either with the weight of a berry, or with the weight of a bunch. The smallest berries were observed in an important must variety

'Rhine Riesling'. This result corresponded with the findings of Fregoni (2005), who indicated that varieties which produce wines of excellent quality generally have smaller berries with higher amount of skin and lower amount of pulp, in comparision with the table varieties. Ojeda *et al.* (2001) pointed out that the size of a berry is an environment-dependent trait. Water deficit that occurs in the early phase from flowering to véraison causes an irreversible decrease in cell volume. Due to changes in water import by xylem, this may lead to a decrease of mesocarp cell turgor (Thomas *et al*., 2006). Under water stress, especially in the early growth stages, the division and elongation of cells decreases (Williams, 2000), thus berries remain smaller. In 2008, when precipitation events were regularly distributed throughout the year, the berries of all nine varieties were larger compared to other observation years.

Sugar content, and thus the quality of the wine, is significantly influenced by the weather, as proven by Grifoni *et al.* (2006) from observations of Italian wines. A surprisingly low sugar content was found in 'Moravian Muscat', despite it being a variety bred in former Czechoslovakia (Pavloušek 1999), and thus adapted to its climatic conditions. However, in this case the sugar content was influenced by the early harvest date, since the grapes were used for the production of federweisser (a freshly fermented must with low alcohol content).

The must yield was also strongly correlated to the size of the berry. According to Fregoni (2005) table varieties have lower must yields than must varieties. However, 'White Chasselas', despite being a table variety, gave the highest must yield during the three study years, while the must varieties, like for instance 'Blue Portugal', produced significantly lower quantities the must (57.00±6.25 ml). This may be the influence of rains right before harvest.

In all eight cases, the artificial neural network was able to recognise all the accessions presented in the learning phase, as published for example by Mancuso (2002) or Mugnai *et al*. (2008). An interesting result was the position of 'Müller Thurgau', which remained separated from all other accessions. Dettweiller *et al.* (2000) confirmed that 'Müller Thurgau' is the descendent of a crossing between 'Madeleine Royal' and 'Rhine Riesling'. Nevertheless, in our results 'Rhine Riesling' remained distant from 'Müller Thurgau', and was assessed as close to 'Sauvignon', corresponding with the data published by Moravcovà *et al*. (2004). As reported in their work (Moravcová *et al*., 2004), 'Red Chasselas' is only a mutation of berry colour of 'White Chasselas'. In the dendrogram, however, 'Red Chasselas' was more closely related to 'Italian Riesling' than to 'White Chasselas'.

Comparing the dendrogram with the PCA score plot, the results were slightly different. Both 'Chasselas' varieties were the closest, which corresponded with the results of Moravcovà *et al*. (2004). But also in this case, there was no evident relationship between 'Rhine Riesling' and 'Müller Thurgau', since they occurred in different quadrants.

Unfortunately, the position of 'Gewürztraminer' in the PCA graph could not be compared with the ANN output. However, the score plot put this variety completely separate from other varieties, e.g. 'Rhine Riesling', as already described by Imazio *et al*. (2002). The great distance of 'Gewürztraminer' from other varieties might also be due to its closer relationship to *Vitis silvestris* (Regner *et al*., 2000; Lacombe *et al*., 2003).

When using the ANN approach it is very important to choose well-developed, healthy leaves (Mugnai *et al*., 2008). An ANN needs a suitable set of sample data to achieve excellent quality in the training set, as this operation can be usually defined as the key point of all the ANN building process (Pandolfi *et al*., 2009 a). Unfortunately, the Euclidean distance did not permit a clear clustering and thus in our case a PCA graph could be considered a more precise tool, even though the data were mostly obtained from subjective observations. However, the possibility of a primary misidentification while still in the vineyard must always be considered: in early spring, when the plants do not have mature leaves or even grapes, an identification error may occur, especially when the varieties are not clearly divided.

Both approaches - ampelography or artificial neural networks – are important methods based on morphological traits, which complement each other to give clear classification of the varieties. The ANN technique represents an more economic alternative to genetic methods commonly used for cultivar discrimination since the equipment necessary for this analysis is inexpensive and commonly available (Pandolfi *et al*., 2009 a).

In this study, application of ANNs led to the distinction of nine selected varieties of *Vitis vinifera*. For the further uses, the ANN profiles obtained from our analysis may be kept in databases for breeding programs, as well as for the description of new cultivars. The data obtained might also be helpful for rapid and reliable identification and description of still unknown varieties, as shown by Pandolfi *et al.* (2009 b).

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