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METHYLEUGENOL AND EUGENOL VARIATION IN OCIMUM BASILICUM CV. GENOVESE GIGANTE GROWN IN GREENHOUSE AND IN VITRO

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INTRODUCTION

The Ocimum genus, collectively called basil, is an important member of the Lamiaceae family. Ocimum includes more than 100 species of herbs and shrubs from the tropical region of Asia, Africa and Central South America (1,2). Sweet basil, O. basilicum, is a common herb cultivated for culinary purposes as fresh, dried or processed. It is an economically important crop in several countries including Italy, particularly in Liguria, where its cultivation covers about 80 hectares. Basil cultivated in Liguria is mostly employed in the preparation of pesto, an Italian sauce known also abroad for its taste and aroma. Among all the basil cultivars, Genovese Gigante is considered one of the best for pesto production, by both producers and consumers. Moreover, only young plants, 10-12 cm in height, are considered to possess organoleptic properties especially suitable for an appetizing pesto: small and young leaves are relished, while plants close to flowering possess a strong, less refined taste. Taste and aroma of each cultivar largely depend on essential oil composition, primarily consisting of monoterpenes and phenylpropanoids (3,4). Although the literature concerning the aromatic composition of basil is significant, very few data are available on the Genovese Gigante cultivar and all refer to the adult or flowering stage (35-40 cm in height). A study of ten Italian cultivars of O. basilicum, analysed at the beginning of the flowering stage, showed that the principal essential oil components were linalool, estragole and cineole, depending on the cultivar (4). The Genovese Gigante cultivar showed linalool as the main component. Since the quality of essential oil is largely influenced by the vegetative stage of the plant, we analysed Genovese Gigante cultivar at the stage in which it is purchased from local markets and used for pesto preparation (5). Methyleugenol was found to be the prevalent component (58.7%) of the essential oil.

Further analyses on basil plants of different heights showed that the main components were methyleugenol and eugenol. Particularly, a correlation between the content of the main components and plant height was found: methyleugenol was predominant in plants up to 6.5 cm, whereas eugenol was prevalent in taller plants (6).

Methyleugenol belongs to the phenylpropanoid group, an important natural constituent of a large number of herbs, spices and vegetables (7-9). Although methyleugenol is approved for commercial use as a flavouring agent in food and as fragrance in perfumes, creams and detergents, human exposure to this compound is of toxicological concern, because of its structural resemblance to known carcinogenic phenylpropanoids, such as estragole and safrole (10). Intake of methyleugenol with the human diet is usually considered to be very low, but the consumption of pesto made with very short basil may lead to the intake of non negligible doses of methyleugenol.

In the present work, our aims were: a) the assessment of methyleugenol/eugenol variation in plants grown in the same place and collected at different age; b) the analysis of the aromatic fraction of basil cultivated in vitro; c) the evaluation of the toxicity of methyleugenol and eugenol using a chicken embryo assay. Furthermore, the distribution of methyleugenol in two parts of the plant was tested.

MATERIALS AND METHODS

Greenhouse cultivation - Seeds of O. basilicum cv Genovese Gigante purchased from SIAS (Società Italiana Agricola Sementi) were sown and grown in greenhouse at 25-27°C, in a suitable soil, generally used in

Liguria for this type of cultivation. Six plants for each different height (10, 20, 30 cm) were harvested and the leaves collected for the distillation. The obtained essential oil was analysed by GC-MS.

"In vitro" cultivation - The same seeds used above were sterilized in 4% NaClO for 15 min, washed three times with sterile deionised water and transferred to Petri dishes with previously damped adsorbent pad. After 4-5 days in the dark, germinated seeds were transferred to MS medium (11) containing 1% agar and 2% sucrose and maintained at 27°C. After 6-8 weeks, leaves from six plants (7-8 cm in height) were collected and processed.

Distillation - In a 500 ml flask, 5 g of leaves from plants cultivated in vitro or in greenhouse were added to 100 ml of water and the mixture was hydrodistilled until 50 ml were recovered. The distillate was extracted three times with freshly distilled ethyl ether. Then the solvent was removed at room temperature and the essential oil was diluted with ethyl acetate to 5 mg/ml. An aliquot was injected into the chromatograph.

GC-MS analysis - Capillary GC-MS measurements were performed on a HP-5MS (0.25 mm x 30 m) column coupled directly to a quadrupole MS. Carrier gas: He; flow rate: 1 ml/min; split 1: 49; injection point: 250°C; oven: initial temperature 60°C for 4 min; ramp: 5°C min^{-1}; final temperature 210°C; electron energy: 70 eV.

Toxicity test - Fertilized eggs of white leghorn were employed. Control and treated eggs were incubated according to the current methods for the same period of time. Stageing of control embryos was made according to Hamburger and Hamilton. Stageing of treated embryos was made according to the number of hours of incubation and referred to the normal stages according to Hamburger and Hamilton. Early embryos were treated at 24 h incubation. Further incubation was carried out up to 50 h incubation or afterwards.

Tested molecules - Methyleugenol and eugenol diluted at different concentrations were separately injected into the eggs, at direct contact with the embryos. Control samples were obtained by injecting the same amount of physiological solution.

Treatment of embryos - In sterile conditions, a window was made in the egg shell, corresponding to the position of the blastodisk. After the treatment, the opening was sealed by a piece of sterile cello-tape. 10 μl of the drugs, used in the range concentration 10^{-2}-10^{-6} M in the physiological solution suggested by Tyrode, were injected under the blastodisk. After the incubation, control and treated embryos were collected, rinsed, fixed and stained for morphological analysis.

RESULTS

We have previously shown that the variation of the main prevalent components of the essential oil in basil plants, collected at the same time from different places, was largely dependent on the plants height, rather than on the age or the growth site. The highest plants analyzed were about 17 cm (6).

The correlation between the variation of methyleugenol and eugenol and the plants' height was also confirmed in this work, where plants 10, 20, 30 cm in height grown in the same greenhouse were analysed. Figure 1 shows the percent decrease in methyleugenol during the growth of plants.

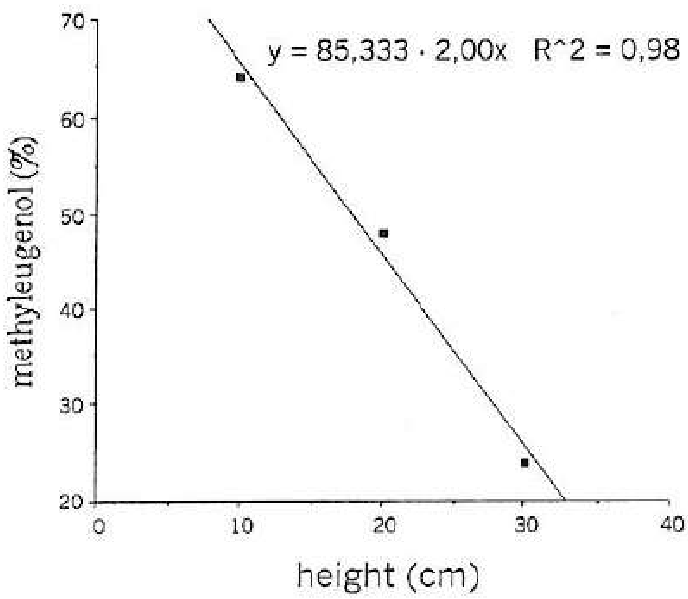


Fig. 1 - Correlation between methyleugenol (%) and plants' height.

The content of methyleugenol and eugenol was also analyzed in plants 7-8 cm in height cultivated "in vitro". The ratio between the two components was not different from that obtained for basil plants of similar height from the greenhouse (fig. 2).

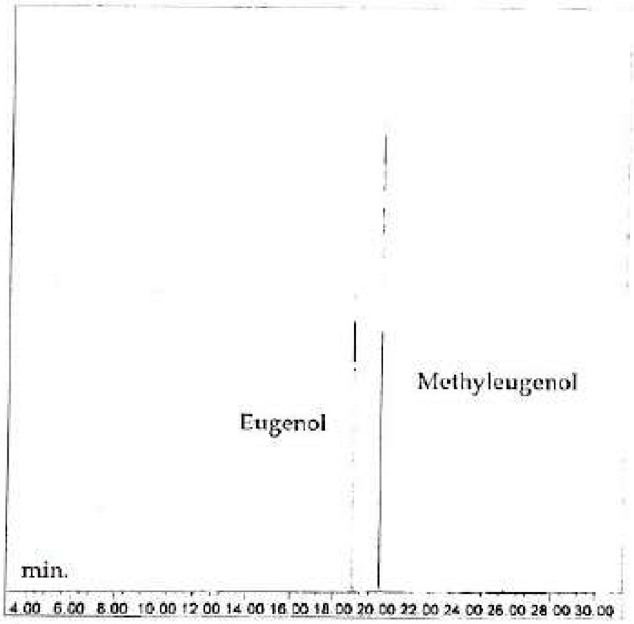


Fig. 2 - Chromatogram of essential oil of basil (7 cm) grown in vitro.

These latter data are of importance in the evaluation of the toxicity of basil containing high percentage of methyleugenol using "in vitro" cell assays, accomplishment of which needs sterile material.
The preliminary results obtained from the analyses of embryos indicate an acute and chronic toxicity of methyleugenol. On the other hand, eugenol does not seem to cause damages to embryos (tab. 1).

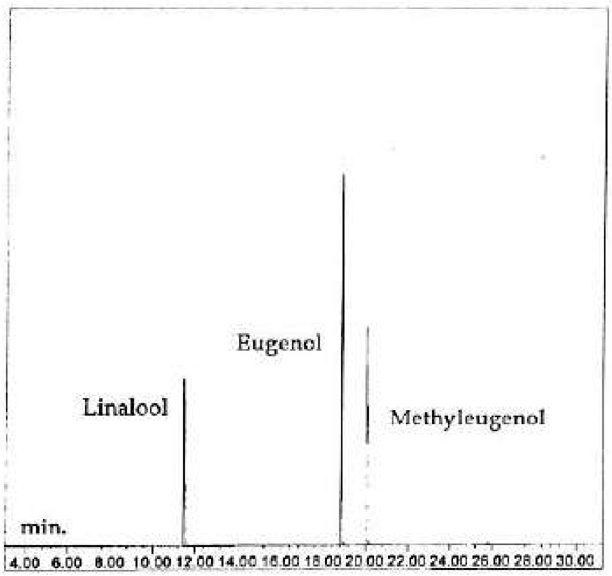
Tab. 1 - Treatment of chicken eggs for 24 hours.

	Methyleugenol Anomalous embryos	Eugenol Anomalous embryos
Samples		
Controls (10)	20%	20%
Treated (20)	65%	15%

DISCUSSION

The data obtained are significant in the assessment of the risk to humans exposed to methyleugenol. NTP reports on the carcinogenicity and toxicity of methyleugenol (12) are generally considered to overestimate the risk to human health posed by dietary ingestion of this compound which is thought to be on the order of a few μg/Kg/day (13). In the case of pesto consumers, intake of methyleugenol could be much higher than that reported above, reaching 250-500 μg/Kg/meal.

Further studies are necessary to evaluate whether a real risk is associated with the consumption of pesto prepared with basil rich in methyleugenol. For this reason, we firstly suggested the use of basil taller than 16 cm, when eugenol is the main component and methyleugenol is greatly reduced. Finally, we have a chance to give a deeper insight at the toxicological concern because we also analyzed different parts of the same plant (20 cm in height): young leaves from the upper part and old leaves from the lower part were separately processed. The decrease in methyleugenol in the upper part clearly results (fig. 3). This is in accordance with our hypothesis on the decreasing activity of the S-adenosylmethyonine-dependent O-methyltransferase during the plants' growth (6). Note that linalool is only present in adult plants (fig. 3).



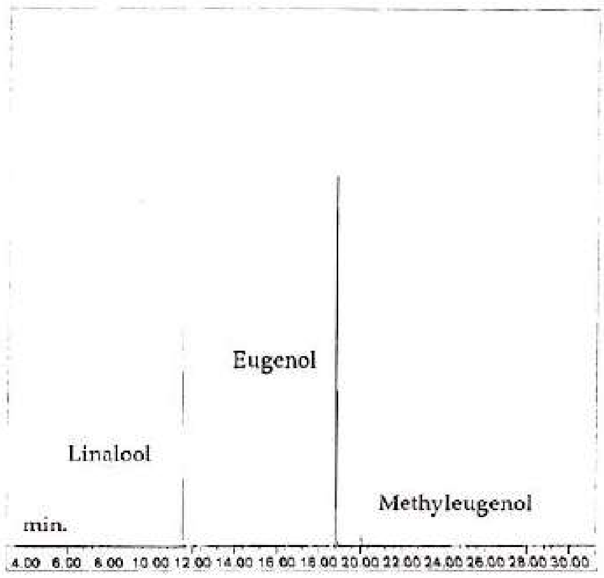


Fig. 3 - Chromatogram of essential oil of basil (20 cm) grown in greenhouse. Left: low part; right: upper part.

In conclusion, we could suggest the use of leaves from the upper part of the plants to ensure the intake of basil with a very low level of methyleugenol.

The variation of the two main aromatic compounds in Ocimum basilicum cv Genovese Gigante grown in greenhouse and "in vitro" was analyzed. The content of methyleugenol and eugenol was correlated to the plants' height rather than to the plants' age and the growth site. Particularly, methyleugenol was prevalent in plants up to 6.5 cm, as plants grew it was replaced by eugenol that was dominant in taller plants. Analysis of basil 20 cm in height showed that methyleugenol is prevalently localized in the low part while eugenol is prevalent in the upper part of the plant. Moreover, a chronic and acute toxicity of methyleugenol was evidentiated in an assay using chicken embryos.

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KEY WORDS: Ocimum basilicum cv Genovese Gigante, methyleugenol, eugenol, toxicity.

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