Agr. Med., Vol. 126, 205-209 (1996)

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SEASONAL TREND OF FLAVONOIDS IN OLIVE (*OLEA EUROPAEA* L.) LEAVES

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SUMMARY - The determination of flavonoids was carried out on olive leaves and five major flavonoids were identified (luteolin, quercitrin, luteolin-7-glucoside, luteolin-4'-glucoside and apigenin). The quantitative determination of these compounds and of chlorogenic acid was carried out for one year in order to study their dynamics in leaves. Three models were found, the first one involving luteolin and luteolin-7-glucoside and exhibiting a spring maximum; the second one involving luteolin-4'-glucoside and chlorogenic acid showing a maximum in early winter, while the third one showed an unchanged trend throughout the whole period.

Key words: *flavonoids, dynamics, olive leaves.*

INTRODUCTION

Flavonoids are widely distributed in plants and seem to play many diverse roles. These products of secondary metabolism may be regarded as biochemical markers and as playing an ecological role in plants; in fact these metabolites are involved in the protection of plants against insects and pathogenes (Harborne, 1988), UV light damage (Graham, 1991), and in the regulation of growth and development (McClure JW, 1975; Wiermann R, 1981).

Flavonoids in leaves of Oleaceae have been investigated by Harborne and Green (1980). In olive, phenolic substances and flavonoid glycosides (Amiot *et al.*, 1986; Vlahov, 1992), as well as glycosides of anthocyanins (Harborne, 1988; Harborne and Mabry, 1980), have been studied in the fruits and oil. However, there is a lack of information about the accumulation of phenolic compounds in the leaves, the primary site of plant metabolism, especially regarding the annual variation of these important compounds.

In a previous paper (Heimler *et al.*, 1992), the quantitative flavonoid content of olive leaves of ten cultivars was determined and minor differences were found among cultivars. We deemed it useful to study their quantitative trend during one year in order to discover the physiological significance. To overcome intraspecific flavonoid variation (Bohm, 1987), a large number of samples was taken and statistical analysis, i.e. 1W-ANOVA (One Way Analysis of Variance), as well as Scheffe's multiple comparisons test were carried out.

MATERIAL AND METHODS

Research studies were carried out on ten mature olive trees of similar age and production in a typical olive-growing area in Tuscany (Pescia 32 m a.s.l.).

The plants were selected in September 1992. Three hundred shoots were labelled in the central part of the canopy of each olive tree.

Three or four shoots per olive tree were collected on 24 October 1991 (period 1) and leaves in the medial zone from these were removed. On eight different dates, during the growth season, this procedure was repeated: 10 December 1991 (period 2), and during the

year 1992, 15 January (period 3), 4 March (period 4), 18 April (period 5), 1 July (period 6), 20 August (period 7), 27 September (period 8), and 29 October (period 9).

Discs of fresh leaf tissue (1 cm diameter) were used for flavonoid and flavone content determination.

The leaves were crushed and kept for 24 hours in 5ml 80% methanol or 70% ethanol; after filtration the solution was spotted on TLC layers. Two kinds of layers with two different eluents were used depending on the polarity of the flavonoids to be separated. Layers of SIL C18-50 (Macheray and Nagel) were eluted with methanol/water/acetic acid (50:50:6); layers of Silica gel 60 F254 (Merck) were eluted with toluene/pyridine/formic acid (100:20:7). Flavonoids were detected as brown spots by UV (254 nm) or were dipped in a methanolic solution containing 1% ethanolamine diphenylborate (NP reagent). Quantitation of spots was carried out with a Shimadzu CS 920 densitometer scanning at 365 nm or 24 hours after dipping the layers in the NP reagent at 440 nm.

The quantitative data are reported as arbitrary integration units since relative variations are more important in this study than absolute values; however all data are referred to leaf dry weight.

The absolute contents of flavonoids lay between 2 and 4 mg/g dry weight for apigenin, 1 and 4 mg/g for luteolin, 1 and 5 mg/g for quercitrin, 1 and 7 mg/g for luteolin-7-glucoside, and 0.1 and 1 for luteolin-4'-glucoside; the water content of the leaves changed from 46% (July) to 57% (April).

Among the 90 samples collected and analyzed, only 88 were included in statistical calculations for luteolin-4'-glucoside because of two missing values, and 86 were included in calculations for luteolin, luteolin-7-glucoside, quercitrin and chlorogenic acid, because of two missing values and two outliers; only 74 observations were included in calculations for apigenin, because of further missing values (among them, those related to the 2nd collecting period). The distributions of the analytical variables considered in this work show very slight departures from normality, and no transformation was needed.

RESULTS

By means of TLC and U.V. spectra the following major flavonoids were identified: luteolin, quercitrin, luteolin-7-glucoside, luteolin-4'-glucoside, and apigenin, which was identified as brown spots under long-wave UV light. Also chlorogenic acid (a caffeic ester of quinic acid) was identified in the flavonoid mixture. In contrast to the findings of Harborne and Green (1980), rutin was not found in the leaves, while the presence of quercitrin was, however, noted. This discrepancy could be ascribed to the choice of cultivars studied, even if intraspecific variation is not supposed to occur.

Flavone aglycones like apigenin and luteolin were found in many herbaceous plants (Harborne, 1980); they are often accumulated externally on leaf surfaces and resins and are encountered very often in plants living in or originating from arid and semi-arid environments and are produced preferentially by plants exhibiting secretory structures (Wollenweber, 1993).

In olive leaves free flavonoids are present, and represent about 35-40% of the whole flavonoid content. Many reports on flavonoid aglycones indicate that these compounds originate from hydrolysis of glycosides during the extraction stage and are not originally present in the plants. In our case, flavonoid aglycones are present in leaf tissues since they are not formed during extraction owing to the kind of solvent and the temperature used (methanol/water mixture at room temperature). In a recent report on olive fruit flavonoids only flavonoid glycosides were identified (Vlahov, 1992).

For each flavonoid, calibration curves were obtained (the correlation coefficients lying between 0.988 and 0.998); however arbitrary integration units were submitted to statistical analysis instead of absolute contents, which may be biased by further uncertainity. The ANOVA F test is not significant for apigenin.

Its mean values do not change in time and show no particular trend. The ANOVA F test is highly significant for the other 5 compounds considered, and the multiple comparison Sheffe test provides further information.

Luteolin (Fig. 1) and luteolin-7-glucoside (Fig. 2) show a maximum in the 5th period (April). This is confirmed by their 5-9 and 5-1 Sheffe's comparisons that are significant at the 0.05 level.

Luteolin-4'-glucoside (Fig. 3) and chlorogenic acid (Fig. 4) show a maximum in the 2nd period (December). Their 2nd period means are different from all the other ones at the 0.05 level. Even quercitrin (Fig. 5) shows a maximum in the 2nd period (December). Its 2nd period means are different at the 0.05 level from the other ones, but not from the 5th period (April) mean.

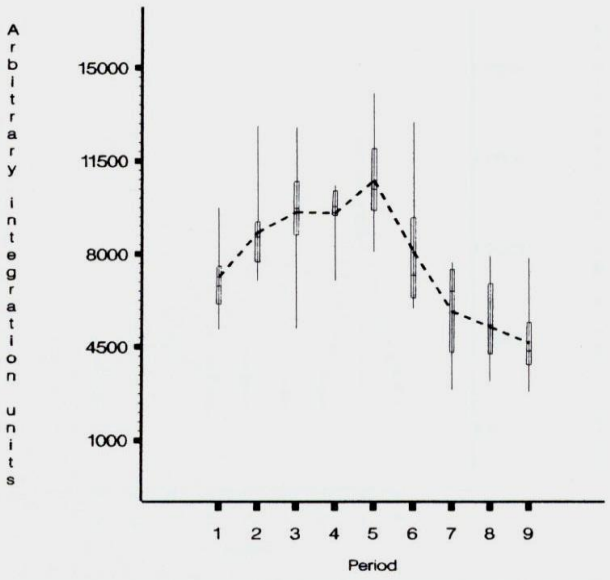

Fig. 1 - Distribution and trend of luteolin. Rectangles are inter-quartile ranges and medians; vertical lines are maximun-minimun ranges. The dashed line links the arithmetic means. For period numbers see the text in the Material and Methods section. A.I.U = Arbitrary Integration Units.

DISCUSSION

Two models can be used, related to time variations of the compounds considered. The first one is characterized by a spring maximum, and involves luteolin and luteolin-7-glucoside leaf contents. The second presents an early winter maximum and it fits the luteolin-4'-glucoside and chlorogenic acid trends. Quercitrin content shows a somewhat intermediate behaviour, while apigenin remains substantially unchanged throughout the entire period.

Only a variation in nitrogen compounds has been studied during a complete annual cycle in olive leaves (Drossopoulos and Niavis, 1988). Nitrogen was found to decrease in spring/early summer and to reach the highest level in autumn/winter period (cit. lit.).

Flavonoid dynamics has been studied in oat leaves where a sharp increase of flavonoids (C-glycosylflavones) accompanied growth and development of leaves and a decrease of flavonoid content was correlated to leaf senescence (Popovici and Weissenbock, 1977); even in pea leaves foliar flavonol concentration decreased with age and senescence (Peyron and Tissut, 1981). In the case of olive leaves, there

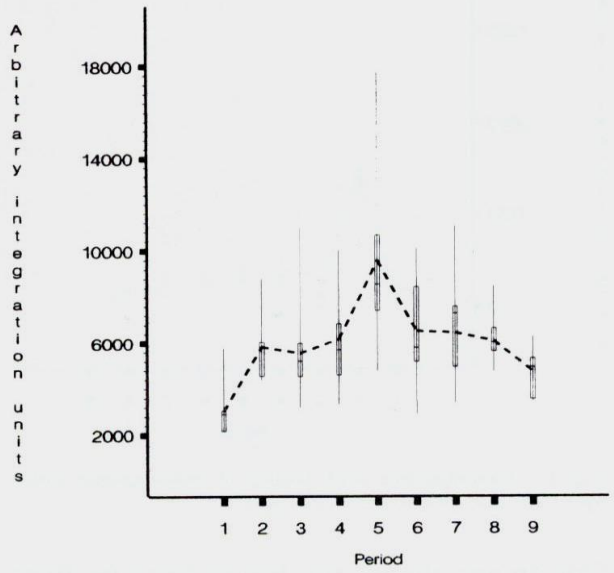

Fig. 2 - Distribution and trend of luteolin-7-glucoside Symbols and numbers as in Figure 1.

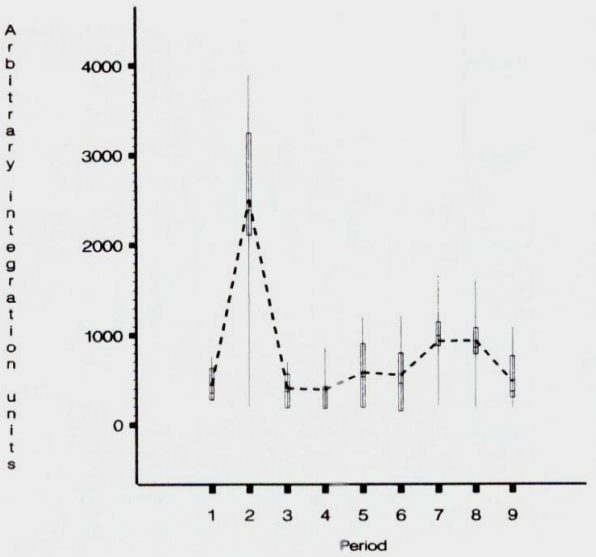

Fig. 3 - Distribution and trend of luteolin-4'-glucoside. Symbols and numbers as in Figure 1.

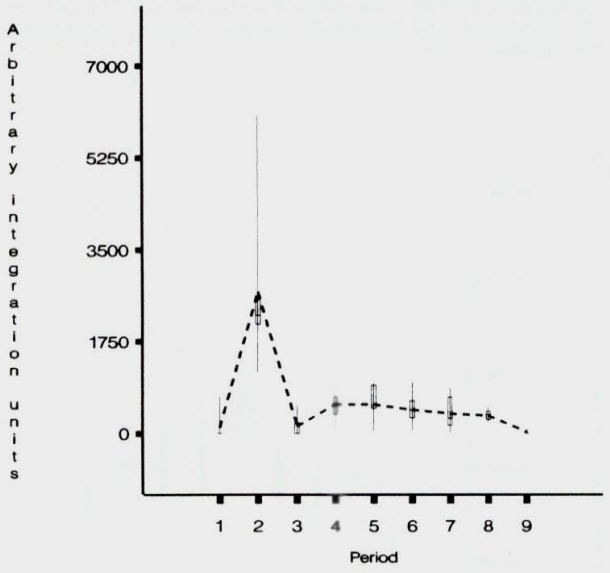

Fig. 4 - Distribution and trend of chlorogenic acid. Symbols and numbers as in Figure 1.

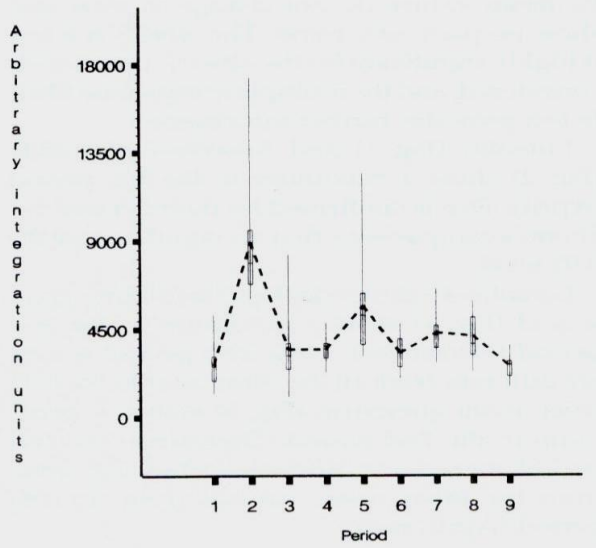

Fig. 5 - Distribution and trend of quercitrin. Symbols and numbers as in Figure 1.

was an age change but leaves were far from senescence, since it was always leaves of that year that were sampled and olive leaves remain for about 1 and half to two years on the tree. An increase of flavonoid content in the spring period can be correlated with the general increase of biological activities at the renewal of the vegetative cycle as already observed in Ginkgo biloba leaves (Lobstein *et al*, 1991). The maximum observed for chlorogenic acid, for luteolin-4'-glucoside and, even if to a lesser extent, for quercitrin is quite difficult to explain. In a paper concerning changes in oleuropeside content of olive leaves during one year, Gonzales *et al.* (1992) found that the iridoid content reached its maximum value in the December-Jannuary period. Chlorogenic acid is one of the precursors of flavonoids in their metabolic pathway and its seasonal variation may be different from that of flavonoids, while the trend of luteolin-4'-glucoside is peculiar with respect to other flavonoids. In fact its content is quite low over the whole year and increases dramatically in the early winter period. This occurrence will be carefully examined in further experiments; however it should be pointed out that glycosidic attachment at the 4' position in the flavone molecule is uncommon in plants (Harborne and Green,

1980) and may perhaps account for the higher reactivity of this compound.

ACKNOWLEDGEMENT

This research was performed with the financial support of Ministero della Universita' e della Ricerca Scientifica e Tecnologica (60%).

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Received: July 1995 *Accepted: November 1995*