Possible juvenile-related proteins in olive tree tissues

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Accepted 9 December 1999

Abstract

Comparisons of protein composition between juvenile and adult organs of olive trees were made by SDS-polyacrylamide gel electrophoresis analysis. Plant material included leaves, bark and bud tissues obtained from juvenile and adult organs within the same plant as well as from separate plants of both the same and different genetic origin. The amount of protein loaded onto the gels was standardized by adjusting the volume of the extract applied to the gel. The protein population of both juvenile and adult tissue from the same or separate trees was qualitatively similar but at least one group of 29 kDa polypeptides, is more abundant in the juvenile leaf tissues. In contrast, a protein group of 35 kDa is more strongly expressed in the adult tissue. Similar relations between the proteins of juvenile and adult tissues were obtained in various cultivars of different genetic origin. Thus, differences in protein composition between juvenile and adult tissues, although only quantitative, are consistent across genotypes. Although, some protein groups, e.g. 63 kDa might be clone-specific some other protein groups also differ quantitatively between adult and juvenile tissues but to a lesser extent. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Juvenility; Protein; Breeding; Olive

1. Introduction

Transition from the juvenile exclusively vegetative to the adult reproductive stage has been referred to as phase change by Brink (1962), ontogenetic aging by

\textit{Abbreviations:} SDS, Sodium Dodecyl Sulfate; PMSF, Phenyl Methyl Sulfonyl Fluoride; PAGE, Polyacrylamide Gel Electrophoresis

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Fortanier and Jonkers (1976), or meristem aging (cyclophysis) by Oleson (1978). This phase change is associated with progressive changes in morphological and developmental attributes and must therefore involve specific gene activation or repression expressed as variations of biochemical factors in the tissues (Hackett, 1987). The length of the juvenile period is influenced by both environmental and genetic factors (Hackett, 1985). Prior to phase change, in woody species flowering does not occur and cannot be induced.

The juvenile non-reproductive phase of developing olive seedlings is characterized by having thick small succulent either round or oval leaves, shoots with short internodes and therefore dense leaf distribution. Juvenile shoot are relatively thick, soft, with a high water content, low lignification and thus a tendency to bend down. Furthermore, all lateral buds on these shoots sprout and develop more such dense vegetative shoots of juvenile nature. The adult form of olive leaves is considerably longer, the leaves are relatively thin and hard with a thick cuticle on their upper surface. The shoots of adult plants are well lignified with rather long internodes. At that phase only a small number of the buds will develop additional vegetative shoots while the majority will differentiate and produce inflorescences.

The leaf polymorphism described is stable and each leaf type —juvenile and adult —goes through a full developmental cycle from young to old without changing their typical morphology.

Leaves of a similar morphology to the juvenile ones on olive seedlings can be found also on very vigorous shoots developing from the base of the trunk of adult trees (suckers). These shoots however, lignify rather rapidly, their internodes are long and only very few lateral buds will sprout and develop vegetative laterals. This type of shoot will usually in less than one growing season develop adult leaf forms. Based on leaf morphology, rate of shoot growth and lack of inflorescence initiation in their lateral buds — such shoots are defined as “pseudo juvenile” or having “secondary juvenility”. The secondary juvenility is unstable while the “true juvenility” of seedlings is stable and considerably longer.

However, little is known about the physiological and metabolic mechanisms responsible for the differences between the juvenile and mature states of cells. Various workers have reported the effect of age on gene expression and particularly photosynthesis genes. Hutchison et al. (1990) showed that the cab gene family is considerably more strongly expressed in light-grown juvenile shoots of larch than in those of adult shoots. They found no difference in the expression of rbc S genes with age. Woo et al. (1994) showed that the in vitro grown petioles of juvenile and adult leaves of Hedera helix responded to NAA differently regarding the expression of HW 101 and HW 103 mRNA. Furthermore Kuo et al. (1995) described a phosphorylation system in Sequoia in which a 32 kDa protein could be phosphorylated only in adult shoots while in the juvenile ones only a 31 kDa proteins was phosphorylated.
Juvenile and adult tissues have been compared physiologically and biochemically by many workers (Jay-Allemand et al., 1988; Monteuuis and Bon, 1990; Murray and Hackett, 1991; Huang et al., 1992). A number of authors have reported differences in protein composition between juvenile and adult plant tissues of different species such as *H. helix* (Fukasawa, 1966), *Sequoiadendron giganteum* (Bon, 1988) and *Castanea sativa* (Amo-Marco et al., 1993). Preliminary results on such differences in the olive (*Olea europaea*) were reported by Lavee et al. (1996).

Changes in protein composition and activity have been described also for many other transition stages during the life cycle of different plant tissues including olives (Lavee and Avidan, 1994). Therefore, standardization and characterization of the plant material used for possible protein changes related to juvenility has to be carefully determined. Identified markers related to the phase change would not only enable evaluation of the developmental stage of the plant but also help in understanding the mechanism involved in this process.

In the present study, we tried to define some possible differences in the protein composition of various organs of juvenile and adult olive plants under well defined genetic and developmental stages. Comparisons of protein composition were made in juvenile and mature organs within the same plant as well as in separate plants from both the same and different genetic origins.

2. Materials and methods

2.1. Plant material

The plant material used in this study originated from four different plant types.
1. Mature flowering cv. Manzanillo trees grown on their own roots with sucker shoots growing from their trunk base.
2. Three- and four-year-old plants belonging to the self and cross pollination F₁ progeny of a breeding program. The plants used were crosses of cvs. Barnea (♀) with Souri (♂) (87-BxS-5, 87-BxS-9, 87-BxS-13), plants from self-pollinated cv. Koronaiki (87-KoS-2, 87-KoS-11) and from self-pollinated cv. Kalamata (87-KaS-14, 87-KaS-19).

All the trees chosen had both shoots showing typical juvenile morphological characters and reproductive flowering shoots. Two-year-old vegetative seedlings derived from seeds of open pollinated cv. Manzanillo trees were also used.
3. Six-year-old plants originating from root suckers of a single plant of a semi-wild olive tree population (gLa). The original plant exhibited juvenile characters although at least 100 year-old due to continuous and massive grazing by goats and sheep. Some of the propagated plants from the original
gLa plant were kept juvenile by continuous severe pruning, while others were grown out of juvenility but part of their branches remained juvenile due to continuous pruning. Another group was developed into fully reproductive mature trees by controlled training and pruning methods.


The tissues analyzed included young and old leaves of polymorphic nature, bark of annually growing shoots and whole in vitro grown plantlets. Sampling was performed separately from the juvenile and mature parts of each tree in most cases during the second half of the summer (July–September).

2.2. Protein extraction

Proteins were extracted according to the method described by Coleman et al. (1991) and modified by us for use with olive tissue. The plant organs collected were rinsed lightly with tap water and immediately immersed and kept in liquid nitrogen. One or two gram tissue were ground within liquid nitrogen in a precooled mortar and pestle. The powder was then homogenized in an Omnimixer with 1:5 w/v of extraction buffer (50 mM sodium borate, 50 mM ascorbic acid, pH 9, 1% β-mercaptoethanol, and 10 mM PMSF). The homogenate was then centrifuged in the cold for 30 min at 35 000 g and the pellet discarded. Proteins in the supernatant were precipitated by adding ice-cold 0.1 M ammonium acetate in methanol. The precipitation was carried out overnight at −20°C. The proteins were collected by a 15 min centrifugation at 10 000 g. The resulting pellet was rinsed three times with 10 ml ice-cold 0.1 M ammonium acetate in methanol and centrifuged as above. Finally, the pellet of proteins was washed once or twice with ice-cold acetone and centrifuged again for 10 min at 10 000 g. The residue was dried under a gentle stream of air and solubilized in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol) and denatured by heating in boiling water for 5 min, then cooled to room temperature and centrifuged for 5 min at 14 000 g. Protein concentration in this final solution was determined according to Bensadoun and Weinstein (1976) with bovine serum albumin as standard. Three to four independent repetitions were performed for each tissue type.

2.3. Electrophoretic analysis

Samples with equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. The amount of protein used was determined within the range 20–50 μg adjusted by the sample volume applied to the stacking gel. Both gradient (15–8%) and non-gradient (10%) running gels with 0.1% SDS, pH 8.3
and a 4% stacking gel were used (Laemmli, 1970). The gradient electrophoresis was performed on 17 cm long gels while the non-gradient ones were only 12 cm long. Electrophoresis was carried out at 20 mA when using the non-gradient gels and at 35 mA when the gradient ones were used. The gel slabs were then stained with 0.25% Coomasie brilliant blue in 40% methanol and 10% acetic acid for about 30 min. In part of the determinations, a staining–fixing solution of 0.125% Coomasie blue 50% methanol and 10% acetic acid was used for overnight staining. The gels were destained by repeated washing in 10% acetic acid and 50 or 40% methanol for 1 or 2 h, respectively. When the staining–fixing solution was used a second destaining solution containing 5% methanol and 1% acetic acid was applied until the gels regained their original size. The reliability of using variable amounts of dissolved protein samples for achieving a uniform concentration on electrophoretic gels was tested. Increasing amount of bark extract from annual growth of juvenile and adult shoots (87-BxS-13) on a single young olive were applied to 10% non-gradient SDS-polyacrylamide gels. A similar experiment was performed also with leaf extracts from adult plants of cv. Manzanillo plants and in vitro plantlets grown from embryos of the same cultivar. Gradient 15–18% acrylamide gels were used. The generality of the protein pattern of juvenile and adults tissue was studied using eight genetically different trees from the F1 progenies of various crosses with both juvenile and adult fruiting shoots on each. Fully developed 8 month-old leaves were sampled from each zone of each tree and protein distribution analyzed and compared. The molecular mass of the protein bands was determined by comparison with reference proteins (RP), ranging from 14.4 to 116.3 kDa (Merck) or 14 to 66 kDa (Sigma).

3. Results

Various amounts of protein were found in the different plant organs analyzed. The reliability of using variable amounts of dissolved protein samples for achieving a uniform concentration on electrophoretic gels was tested. 30, 40 and 50 µl of bark extracts from annual growth of juvenile and adult 87-BxS-13 olive plants were applied to SDS-polyacrylamide gels. The protein distribution within either the adult or juvenile bark of a single plant was the same at the three concentrations applied, showing an increase in protein band intensity respective in both cases (Fig. 1). Thus, within the range of protein concentrations in our extracts (300–1000 µg ml) the amount of protein applied to the gels could be standardized by adjusting the amount of extract loaded in the stacking gel. The protein content of the two extracts, from juvenile and adult bark, used in this experiment was about the same (420±17 and 430±21 µg g⁻¹, respectively).
A major protein group was found at ca. 35 kDa which was more strongly expressed in the bark extracts of the adult tree part of the 87-BxS-13 clone. The other distinct groups at 29, 61 and 63 kDa were more expressed in the bark of the juvenile tree part.

A similar experiment was conducted with leaf tissue. In this case, 20, 40 and 60 μg protein extracted from adult leaves of cv. Manzanillo and from leaves of in vitro germinated embryos of the same cultivar were applied to 15–8% gradient gels. A uniform protein distribution and a clear concentration response were noticed also within each of the two leaf extracts. Two clear bands of protein with a mass of about 55 and 14 kDa were noticed in both samples (Fig. 2) and were quantitatively similarly expressed in both the extracts of juvenile and adult leaves. However, a marked quantitative difference was found for a protein with a size of ca. 30 kDa. This protein was expressed in extracts of the juvenile leaves from the in vitro grown plants and appeared only very weakly in the extracts of adult leaves of cv. Manzanillo. In the case of cv. Manzanillo another protein more expressed in the juvenile than in adult leaf extract had a mass of ca. 63 kDa. This however, seems to be cultivar-dependent as it is expressed in some clones and not in others and was also not expressed in buds. A ca. 35 kDa protein was clearly more expressed at the two higher concentrations of the adult leaf extracts. The drawback of the experiment with the leaf extracts was the genetic variation between the adult (cv. Manzanillo) and juvenile (in vitro grown cv. Manzanillo embryos) plants used.

The nature of protein distribution was determined in buds, leaves and bark of juvenile and adult plant sections of annual shoots of a cv. Barnea ♀ Souri ♂ cross (87-BxS-13). Although the organs used originated from the same tree,
considerable differences in protein constituents of the various organs were apparent. The differences in proteins between organs were considerably larger than those between similar tissue taken from juvenile and adult plant parts (Fig. 3). While some qualitative differences in protein were apparent between organs, mostly quantitative ones could be noted between similar tissues derived from juvenile and adult plant parts. In bark, this quantitative difference in protein expression between the juvenile and adult tissues was strongly apparent with the

![Image](image1.png)

Fig. 2. Distribution of proteins in the leaves of adult potted cv. Manzanillo plants (A) and of in vitro germinated cv. Manzanillo seeds (J) on a gradient 15–8% SDS-AA gel loaded with three amounts of protein for each sample.

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![Image](image2.png)

Fig. 3. Protein distribution in extracts of juvenile and adult buds, leaves and bark of the 87-BxS-13 olive plant (Barnea ♀ × Souri ♂ F₁) (non-gradient 10% SDS-AA gel; samples applied contained 40 µg protein).
35 kDa proteins which were considerably more expressed in the adult tissue than in the juvenile one.

Major differences in protein distribution were found between young and 1 year-old mature leaves, when both were derived from juvenile and adult shoots of an F₁ progeny tree (87-KoS-2) from self-pollinated cv. Koronaiki (Fig. 4). A considerably larger number of proteins of all sizes were visible in the extracts of the fully expanded mature leaves than in those of young half expanded ones. This difference was particularly expressed with the smaller sized protein groups.

The total amount of protein on tissue fresh weight basis was significantly higher in the aged leaves of both juvenile (750±49 μg g⁻¹) and adult (950±65 μg g⁻¹) origin than in the respective young ones (375±38 and 450±33 μg g⁻¹). In this 87-KoS-2 clone, in both leaf age stages, the respective amount of protein in the extracts of the adult leaves was about double of that found in the juvenile ones. With most clones tested this difference in protein content between juvenile and adult tissue was smaller, ranging 20–40%.

The juvenile and adult olive leaves are highly polymorphic and thus very different in form (Fig. 5). Still, no differences were visible in the protein groups of young leaves deriving from juvenile and adult parts of the same tree. In fully mature leaves quantitative differences were noted mainly in three protein groups between juvenile and adult tree parts.

Proteins of ca. 29 and 63 kDa were more expressed in extracts of leaves from the juvenile plants than in leaves from the adult trees though in young leaves this was less clear. The protein with a mass of ca. 35 kDa was more expressed in the extracts of adult leaves when all the leaves used were of similar developmental age (Figs. 2 and 4).
In order to avoid genetic diversity and achieve high protein specificity, juvenile and adult samples were taken primarily from organs and tissues of the same tree. The metabolic independence of the juvenile and adult tissues of the same tree was verified by comparing similar tissues derived from separate adult and juvenile trees or a tree with both juvenile and adult branches, all from the same genetic origin (clone gLa).

The protein population of the juvenile and adult tissues from separate or the same trees was identical (Fig. 6). The differences between the juvenile and adult tissues were quantitative and similar to those described above for the leaves of cv. Manzanillo and the 87-KoS-2 clone. Still in this case the 63 kDa protein was more expressed in the adult leaves.

A comparison between the protein distribution in leaves of juvenile in vitro germinated seedlings with that of semi-juvenile leaves from suckers and adult leaves of a cv. Manzanillo tree was made on gradient gels. Two proteins of 55 and 63 kDa were more expressed in the adult leaves.
14 kDa mass were clearly expressed in all extracts of the leaves (Fig. 7). Based on their mass, these two proteins already shown earlier in both adult and juvenile leaves (Fig. 2) correspond with the published masses of Rubisco present only in CO₂ fixing tissues. The 29 kDa protein that is more expressed in the juvenile leaves was less apparent in the extracts of sucker leaves which are considered of secondary juvenility. In this experiment two other proteins of 82 and 78 kDa
were quantitatively more apparent in the extracts of the true juvenile leaves, however, this was not so in all replicates. The total amount of protein mass on fresh weight basis was within the range 500–750 μg g\(^{-1}\) for both the juvenile and adult leaves of eight different clones tested, without significant difference between them (the mean was 658±41 and 680±39 μg g\(^{-1}\), respectively). The protein distribution showed similar relation between the juvenile and adult leaves in all the clones (Fig. 8). However, the level of expression was different for the various proteins within each clone. In all the eight clones, a protein band corresponding to 29 kDa was more expressed in the juvenile leaves than in those from the adult reproductive zone of the tree. Proteins of 35 kDa were usually more expressed in adult leaves while the 63 kDa protein level was not consistent and seemed clone specific. Other clone specific differences were also apparent, though the distribution of proteins in the eight clones tested was rather similar.

4. Discussion

Polypeptides with a molecular mass of ca. 29 kDa seem to be associated with juvenility in the olive. These proteins were considerably more conspicuous in the extracts obtained from leaves or plantlets in the juvenile phase. These protein exist also in the adult tissues but are much less expressed. The difference between juvenile and adult tissues might be only quantitative. Similarly, proteins corresponding to 35 and 63 kDa are strongly expressed in the adult tissue but present to a lesser extent also in juvenile tissue. These results differ from those obtained with other species. Bon (1988) reported a membrane-associated protein known as J16 (16 kDa), present in juvenile S. giganteum tissue but absent in mature clones. In Sequoia sempervirens (Huang et al., 1992) eight proteins were described which could be detected in either adult or juvenile leaves but not in both. Also, Amo-Marco et al. (1993) observed differences between the polypeptide content of in vitro shoot cultures derived from upper branches (adult) and basal shoots (juvenile) of the same chestnut tree (C. sativa). Cultures derived from mature crown growth yielded two polypeptide bands (43.6 and 38.0 kDa) which were not found in cultures from juvenile basal shoots.

For the olive tree, little information on protein distribution and changes is available. Lavee and Avidan (1994) reported in relation to alternate bearing, differences in the protein content and composition of “on” and “off” year olive tree leaves and bark of one-year-old shoots. Recently, Tazzari et al. (1995) found differences in a 66 kDa protein that was present in the leaves of adult upper branches of an olive tree but not in those of suckers. In the present study we did not find a 66 kDa protein in olive tissues but a somewhat smaller one of 63 kDa.
was very apparent. This protein however, was not consistent in its expression between juvenile and adult leaves of the various clones and seems clone specific. It should be noted that this protein has also not been detected in leaves of suckers of the same tree. The absence of this protein only from the extracts of sucker leaves, a semi-juvenile tissue, and not in the leaves of truly 2 year-old juvenile seedlings might result from some physiological characteristic, specific for the sucker tissue, growing from the base of the tree trunk. Similarly, Huang et al. (1996) have shown that by repetitive grafting of adult sequoia shoot tips on juvenile rootstocks, they induced rejuvenation, restoring the rooting ability of the adult shoots. This rejuvenation was accompanied by the disappearance of adult associated esterase and peroxidase isozymes and appearance of those characteristic for the juvenile phase of the shoots.

However, a quantitative difference between the expression of such a protein was found also in extracts of adult and truly juvenile tissues. A protein of ca. 29 kDa was usually more expressed in extracts of all juvenile leaves bark and buds tested. However, it was present also in the same tissues of adult origin. Although extracts of different tissues, leaves, annual bark and buds show a different protein distribution, most differences in protein expression between juvenile and adult tissues were more or less apparent in extracts of all tissue types. In mature leaves of both juvenile and adult origin two proteins with a molecular mass of 55 and 14 kDa were equally expressed. These two proteins, found only in the leaf extracts, corresponded with the small and large subunits of Rubisco as described by Hubbs and Roy (1992) working with pea chloroplasts and using a similar analytical system.

Complete expression of juvenile proteins on mature trees has, so far, been observed only in tissues deriving from a sexual process (Bonga, 1987). In olive, however, tree juvenility can remain for many years in portions of adult trees as is expressed morphologically by the growth habit of shoots and leaves. The existence of proteins associated with either of the two, juvenile and adult phase on the same tree, should be indicative of a genetically programmed phase change resulting in a physiological transition in the metabolism of the two phases (Meier-Dinkel and Kleinschmidt, 1990). This was quantitatively apparent also on the total protein on fresh mass basis which was in most cases significantly higher in both young and mature adult leaves than in the respective juvenile ones.

References


