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Full Length Research Paper

In vitro vegetative growth and flowering of olive tree in response to GA3 treatment

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The phytohormone gibberellin is involved in the regulation of many physiological process including flower induction and shoot growth. In this study, gibberellic acid (GA3) was used in order to induce the reversion of olive tree vegetative buds towards a floral ones *in vitro*. For this, six varieties (Marsaline, Chemchali, Picholine, Chemlali, Zalmati and Oueslati) was tested and explants, consisting of a single node segments, were grown in media containing three concentrations of GA3 (1, 2 and 10 mg/l). Results show that Marsaline seems to be the most able variety in regenerating floral structures. For this variety, 5 cases of reversion were observed mainly on the medium containing 10 mg/l GA3. This same medium was also favorable for this transformation for the other varieties (3 cases on Picholine, one each on Chemchali , Zalmati and Oueslati). The examination of the histological sections confirmed this transformation. In addition, this experiment showed that GA3 can be at the origin of an interesting growth rate of vegetative buds, which elongation depend on variety as well as GA3 concentration.

Key words: Olive tree, *in vitro* flowering , GA3, reversion.

INTRODUCTION

The gibberellins are involved in several physiological process regulations such as seed germination, flower induction and development and shoot elongation. Ameha et al. (1998) reported that flowering of *Cucumis* seedlings was regulated by gibberellin, while an important accumulation of GAs during the flowering transition was found in the petioles of *Arabidopsis* by Gocal et al. (2001). Ben Nissan et al. (2004) observed that GIP1 expression (which is a protein induced by GA3) coincided with cell elongation in stem and flowers transition in *Petunia hybryda*. Mistra and Datta (2001) revealed a significant role of GA in the induction of shoots buds on leaf segments of Marigold, and Hall and Camper (2002) successfully used GA3 to develop an *in vitro* culture protocol for *Goldenseal* species.

However, the *in vitro* flower-bud induction is still rare. It occurs only under special conditions. This induction depends on several features such as genetic, hormonal and trophic factors and seemed to be the result of the repression of growth genes and the activation of those responsible of flowering process. *In vitro* flowering of various crops has already been accomplished but depends on differences in explants type, growth regulator, composition of media (auxins, cytokinins, gibberellic acids) and culture environment like temperature and photoperiod.

For olive tree, the ability to control *in vitro* flowering could facilitate repeated cycles of crossing and accelerate genetic improvement programs. It is well

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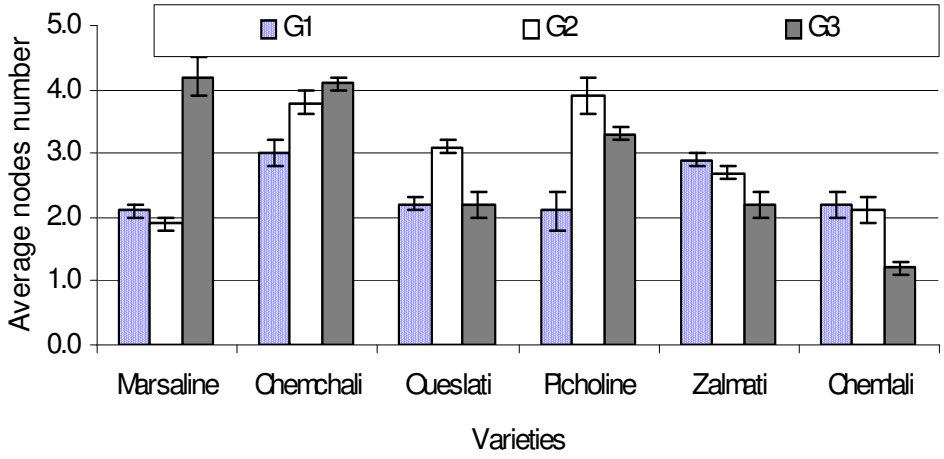

Figure 1. Average of the shoots' development on G1, G2 and G3 media.

known that olive tree seedlings, produce the first flowers after 15 to 20 years after planting (Fontanazza, 1997). Several research demonstrated that the application of GA3 on olive trees increased the number of perfect flowers *in vivo* (Mehri et al., 1995). In this paper, *in vitro* vegetative growth and appearance, for the first time, of flowering structure on subcultured olive tree shoots are reported. In addition, anatomical and histological characteristics of the flower structure initiation were examined.

MATERIAL AND METHODS

Vegetative material and *in vitro* method

Seedling plantlets of six Tunisian olive tree cultivars (Marsaline, Picholine, Chemlali, Zalmati, Oueslati and Chemchali) were initiated on OM medium (Rugini, 1984). After 2 years of subculturing, nodal segments (1 cm long with 2 axillary buds) of each cultivar were used for flower induction experiment. Explants were placed on a basic media containing a half of OM (Rugini, 1984) and half MS media (Murashigue and Skoog,1962) nutrients (macro and micro) supplemented by the vitamins of OM medium. Three concentrations of GA3 were added to the basic media: 1 mg l^{-1} (for G1 medium), 2 mg l^{-1} (for G2 medium) and 10 mg l^{-1} (for G3 medium). 48 explants per media were used. Media were dispensed into glass tubes (20 ml) and autoclaved for 20 min at 120°C. Cultures were grown at 24 ± 2°C in a 16 hours photoperiod supplied by white fluorescent light (3000 lux). Appeared nodes and flower structures were recorded periodically.

Histological technique

Vegetative and floral tissues were first fixed in Navashine solution made up of chromium (IV) oxide (10 volumes), formaldehyde (4 volumes) and acetic acid (1 volume) for 48 h. The tissues were dehydrated afterwards in an ethanol-xylen mixture. Sections of 10 µm were obtained by a Leica microtome and then colored by hematoxin and eosin green light. Sections were observed and photographed in a wide microscope.

RESULTS

Vegetative growth and rooting

Maximum growth was noted at the end of three months of culture. The number of appeared nodes from each explant was variable according to the variety and the culture medium. An average of more than 4 nodes for both Marsaline and Chemchali on G3 (10 mg l^{-1} GA3) was observed whereas under 3 nodes for Chemlali and Zalmati on all media was recorded (Figure 1). For Picholine and Oueslati, the maximum of growth was recorded on G2 medium (2 mg l^{-1} GA3) were 3.9 and 3.1 new nodes, respectively. Chemchali seemed to be the best variety in the sense that it provided high perfomances on all media.

After the period of three months, no more growth was observed and yellowing was visible on some shoots which is probably due to the impoverishment of the culture media. After 4 to 5 months, formation of roots occur as indicated in Table 1. The juvenility of the material and the impoverishment of the culture media seemed to be at the origin of the root appearance which might limit *in vitro* flowering reversion.

Floral induction

The floral structures appeared at the end of 3 months of culture; 4 for Marsaline (1 on G1, 1 on G2 and 2 on G3), and one each for Chemchali and Oueslati on G3 and G2 on G3 media (Table 2). After 6 months, another floral structure was observed in Marsaline variety and after 9 months, 3 new floral structures for Picholine and one for Zalmati were seen. Floral structures were not observed in Chemlali. The transfer of some cultures to a new media containing zeatin (1 mg/l) allowed a significant vegetative

Table 1. Rate (or percentage) of roots formation by the varieties in the three media.

Cultivars	G1 (1 mg l^{-1} GA3)	G2 (2 mg l^{-1} GA3)	G3 (10 mg l^{-1} GA3)
Marsaline	33	40	62
Chemchali	40	77	0
Oueslati	23	43	25
Picholine	0	42	48
Zalmati	50	59	29
Chemlali	33	33	11

Table 2. Floral structures number formed by the different olive tree varieties on the different media after 3, 6 and 9 months (3, 6 and 9 M).

Cultivars	G1 (1 mg l^{-1} GA3)			G2 (1 mg l^{-1} GA3)			G3 (10 mg l^{-1} GA3)			Total
	3 M	6M	9M	3 M	6M	9M	3 M	6M	9M	
Marsaline	1	0	0	1	0	0	2	1	0	5
Chemchali	0	0	0	0	0	0	1	0	0	1
Oueslati	0	0	0	1	0	0	0	0	0	1
Picholine	0	0	0	0	0	0	0	0	3	3
Zalmati	0	0	0	0	0	0	0	0	1	1
Chemlali	0	0	0	0	0	0	0	0	0	0
Total	1	0	0	2	0	0	3	1	4	11

Figure 2. External morphology of the floral buds. Bar: 2 mm.

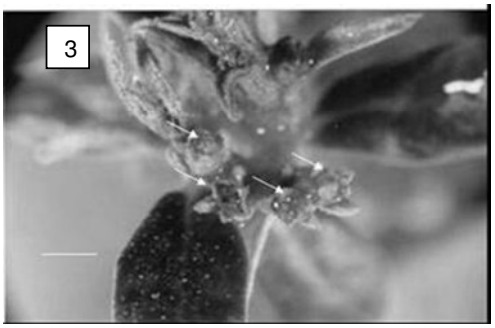

Figure 3. A cluster of floral buds. Bar: 2 mm.

development without appearance of new floralistructures. The floral structures induction was presented in the form of a transformation in the buds morphology, which was initially leafy. Its tips expanded with a slight flatness (Figure 2). The induction also allowed the appearance of small pieces reminding bracts and sepals. In general, single flower was observed (Figure 2) except in only one case where a cluster of flowers was formed (Figure 3). The comparison of the histological sections done on vegetative buds and those made on the transformed ones allowed for distinguishing clear differences between the 2 types of structures (Figure 4 and 5). In fact, on the induced structures, three protuberances of which the central one was the largest (Figure 4) were visible.

The cytoplasm of these cells, mainly for lateral formation, appeared very dense. This state corresponded exa-

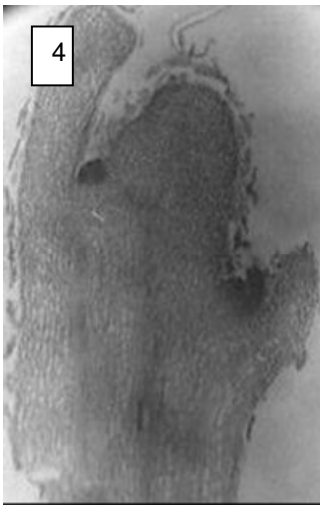

Figure 4. Histological section of the floral structure, x 50.

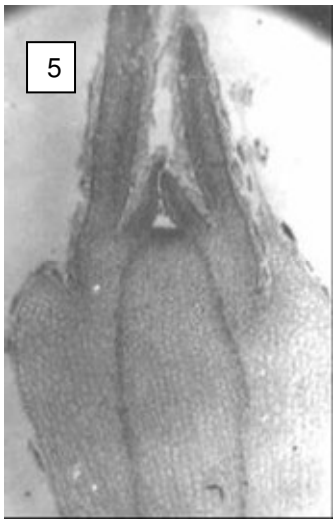

Figure 5. Histological section of vegetative bud, x 50.

ctly to the first flower formation (the first stage of olive tree inflorescence appearance *ex vitro*) and the two secondary meristems to the second stage (with 2 flowers). This presents, in general, the differentiation step of flower ontogenic process in olive tree and *in vitro* flower structures were morphologically comparable to *in vivo* ones at the first stage. However, keeping these structures on their initiation media or their transfer to new ones did not allow the structuring of normal and complete flowers. In all cases, necroses of these formations were noted. In addition, the appearance of roots seemed to inhibit the formation of more floral structures.

DISCUSSION

The results above demonstrated that GA3 can play an important role on *in vitro* olive tree vegetative growth and on its *in vitro* flowering as well. This is one of the rare occasions where it is reported that GA3 can have an influence on *in vitro* olive tree growth because most of searchers indicated the necessity to add at least 1 mg/l of zeatin to the culture media in order to obtain a high multiplication rate for this species (Rugini 1984; Chaari-Rkhis et al., 1997, 2002, 2003). However, GA3 was succesfully used by Dimassi-Theriou (1994) and Grigoriadou et al. (2002) combined with BA or Zeatin, respectively, for shoot proliferation of certain olive tree cultivars. In reality, GA3 is an interesting hormone for *in vitro* shoot elongation of many other species such as *Macadamia* (Mulwa and Bhalla, 2000), *Acacia* (Vengadesan et al., 2003) and *Quercus* (Purohit et al., 2002). In this investigation, media containing GA3 at a variable concentrations and a weak concentration of zeatin (0.1 mg/l) provide, mainly for the Chemchali, Marsaline and Picholine, a relative high multiplication rate. Using 1 mg/l of Zeatin by Chaari-Rkhis et al (2003) enhance notably *in vitro* multiplication rate of Chemchali and Marsaline, reaching more than 10 new nodes after three months of culture.

It is important to note the appearance of roots at the end of the culture. This phenomenon can, probably, be attributed to the long-established culture or to the presence of GA3 in the medium. GA3 is one of the hormones able to induce *in vitro* rooting (Zrÿd, 1988).

According to our results*, in vitro* flowering structures could be successfully induced in olive tree seedling by gibberellic acid addition in the culture medium. There is a strong relationship between GA3 concentration and flowering structure number. The majority of flower struc-tures was developed after 3 months of culture (6/11 cases). It is important to remark the varietal differences in the number of floral structure regeneration. The floral structure induction may be explained by the impoverish-ment of the culture media. In fact, many kind of stress can be at the origin of flowers formation. Thorpe (1980) and Neelu (1997) report that stress can enhance *in vitro* flowering.

Flower structure initiated was manifested by changes in the size and shape of the apical shoot bud which underwent transition from a vegetative state to a reproductive one. The histological sections show bracts and 2 types of meristems identical with those observed at the beginning of the normal inflorescence formation. In fact, Vallade (1999) indicate that, in general, the first step of the inflorescence apparition was the stucturation of the inflorescentiel meristem and two lateral meristems. The comparison of the sections done on the structure induced *in vitro* with those carried out on olive normal flowers taken on adult trees show a great similarity. This morphological stage corresponds to the stage C on olive tree ontogenesis according the flowering stages defined by P. Colbrant and P. Fable (Loussert and Brousse, 1978). Hartmann (1951) and Fabbri and Alerci (1999)

noted the same morphology on the inflorescential ontogenesis of some olive tree cultivars. In our case, the transformation is stopped at this level. In general, floral induction on olive tree occurs following a cooling winter (Fontanazza, 1997; Lavee and Harshemesh, 1986). The results presented above suggest that gibberellic acid can play an important role in the induction of flowering process in olive tree. This implies that the effect of the winter cold might be replaced by the aibberellic acid action. However, gibberellin promote, in general, flowering of the long days plants as report by Pharis and King (1985). These workers also observed that, for woody angiosperms, application of GAs often inhibits flowering.

Much progress in understanding flowering transition in plant was accomplished because of the numerous research on *Arabidopsis* (Vallade, 1999). Gocal et al. (2001) find that the state of transition to flowering in *Arabidopsis* was accompanied by an accumulation of GAs in its petioles. Blazquez et al. (1998) provide evidence that the expression of the floral gene LEAFY was correlate with endogenous gibberellins level. Ameha et al. (1998) demonstrated the *in vitro* implication of gibberellins in flowering transition of cucumis seedlings. While, Chen et al. (2003) indicated that for Philodendron both growth and flower number per plant increased as GA3 concentration increased from 125 to 1,000 mg/l.

For olive tree, this is the first time that flower structure was initiated *in vitro* on this species. But the induced flowers seemed to be abnormal because all of them manifested necrosis and finally died. The same manifestation was reported by Lavee and Harshemesh (1986) working on juvenile olive plant. In our trial, the low rate of those obtained floral structures probably has its origin in the appearance of roots. In fact, some researchers indicated that there is an antagonism between *in vitro* rooting and flowering (Ammar et al., 1987). Flowering *in vitro* has been successful in many species using other conditions and products such as N-(2-chloro-4-pyridyl)-N'-phenylurea and cold on *Pyrus communis* (Harada and Murai, 1998), kinetin on *Gentian* (Zhang and Leung, 2000) and thidiazuron on *Dendrocalamus strictus* (Singh et al., 2000).

In conclusion, GA3 can play an important role in the transition from a vegetative to a floral state but there is certainly other biochemical and environmental factors involved in this process.

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