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Full Length Research Paper

Influence of fertilization and drought stress on the growth and production of secondary metabolites in *Prunella vulgaris* L.

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The combined effects of fertilization and drought stress on the growth of *Prunella vulgaris* and the production of rosmarinic acid (RA), ursolic acid (UA) and oleanolic acid (OA) were investigated. The factors studied included two water conditions and three fertilizer regimes. The results showed that moderate drought stress dramatically decreases spica biomass production but increases the content of RA, UA and OA in spicas of *P. vulgaris*. Total RA, UA and OA yields were found to be significantly higher in well-watered plants than in drought-stressed plants. An appropriate amount of fertilizer could alleviate the negative effect of drought stress on the growth of *P. vulgaris* and its production of RA, UA, and OA. The interaction of water and fertilizer treatments significantly influences vegetative dry weight, reproductive dry weight and total RA, UA and OA yields in *P. vulgaris.* The results suggest that the application of the proper amount of fertilizer aids *P. vulgaris* production in arid and semi-arid regions and that the combined use of fertilizer and properly timed exposure to drought stress can enhance total RA, UA and OA yields in *P. vulgaris.*

Key words: *Prunella vulgaris* L., N, P and K fertilizer, growth, drought, secondary metabolites.

INTRODUCTION

Prunella vulgaris L. (Labiatae), also known as the “self-heal,” is a perennial herb commonly found in North Asia, Europe and North Africa. The dried spica of *P. vulgaris*, Prunellae Spica, a standard medicinal material in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), has been used as a traditional medicine in China for over a hundred years (Chen et al., 2010). *P. vulgaris* has also been used as a herbal medicine to alleviate fever, reduce sore throat, and accelerate wound healing (Pinkas et al., 1994; Psotová et al., 2003, 2006). *P. vulgaris* is rich in phenolic acids. One of the major phenolics, rosmarinic acid (RA) (Psotova et al., 2006), suppresses lipoperoxidation (Laranjinha et al., 1994), scavenges superoxide radicals (Osakabe et al., 2002), and exhibits anti-inflammatory (Osakabe et al., 2004) and antioxidant (Psotova et al., 2006) bioactivity. Rosmarinic acid (RA) has also been used as the standard for quality control of Prunellae Spica by the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010).

Triterpenes are the dominant compounds in *P. vulgaris* (Cheung and Zhang, 2008). Of the triterpenes, ursolic acid (UA) and oleanolic acid (OA) are especially known for their many bioactivities, including hepatoprotection, antihyperglucemia, antifugal, anti-tumor and anti-inflammatory activities (Liu, 1995). In addition to its pharmaceutical uses, the spicas of *P. vulgaris* are manufactured as a refrigerated beverage, and the fresh leaves of *P. vulgaris* are consumed as a vegetable dish in southeast China.

Due to its medicinal and industrial importance, the

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demand for *P. vulgaris* has steadily increased in the world market. The wild population of *P. vulgaris* cannot meet this growing need, and therefore it has been proposed since the 1990s that *P. vulgaris* be cultivated to allow for more efficient resource utilization in China. The cultivation of medicinal plants could also curtail excessive harvesting of wild resources, which leads to loss of genetic diversity and habitat destruction (Canter et al., 2005). Field plantations are the major source of *P. vulgaris* spicas; harvests are usually done from May to mid-June annually (Chen et al., 2010). A rational agronomic practice of *P. vulgaris* is necessary to meet the world market demand for uniform and high quality raw material.

Numerous studies have shown that environmental stress enhances the production of several secondary metabolites by plants (Zobayed et al., 2005; Jaleel et al., 2007a). Thus, the production of high levels of secondary metabolites can be induced in plants by controlling certain environmental factors (e.g., specific stress conditions) (Zobayed et al., 2005, 2007). For instance, the accumulation of secondary metabolites can be induced with exposure to nutrient deficiency (Stewart et al., 2001), UV light (Winkel-Shirley, 2002), light intensity (Mosaleeyanon et al., 2005) and high temperature (Couceiro et al., 2006). Drought is one of the most important environmental stresses that can depress the growth and alter the biochemical properties of plants (Zobayed et al. 2007). This type of stress is known to increase the amount of secondary metabolites in a variety of medicinal plants, e.g., artemisinin in leaves of *Artemisia annua* L. (Charles et al., 1993), hyperforin in *Hypericum perforatum* leaf tissues (Zobayed et al., 2007) and ajmalicine in *Catharanthus roseus* roots (Jaleel et al., 2008).

In general, drought stress strongly inhibits plant growth and development, but the application of suitable fertilizers can alleviate these effects (Wu et al., 2008; Zhu et al., 2009).

The combined effects of drought stress and fertilization on medicinal plants are important for the development of field cultivation. But little is known about this topic for *P. vulgaris*. The objectives of this study were to determine the combined effect of drought stress and fertilizer on the growth and RA, UA, and OA production in spicas of *P. vulgaris.*

MATERIALS AND METHODS

Plant growth and treatment

An experiment using potted plants was conducted from October 2009 to June 2010 in a rain shelter located at the Institute of Chinese Medicinal Materials, Nanjing Agricultural University, Nanjing, Jiangsu Province, P.R.China. Surface soil from an experimental field in Nanjing Agricultural University was used as the growth substrate.

The soil collections were combined and thoroughly mixed. Soil (4.0 kg) was placed in each of 4.5 L plastic pots. The organic matter content of the soil was 21.32 $g \cdot kg^{-1}$; available-N was 34.65 $g \cdot kg^{-1}$; available-P was 12.07 $g \cdot kg^{-1}$ and available-K_2O was 16.34 $g \cdot kg^{-1}$. The water field capacity was 25%.

We designed a randomized experiment consisting of two water regimens 70 to 75% and 45 to 50% of field water capacity (FWC) and three fertilizer treatments (N0P0K0 = without fertilization (control), N1P1K1 = 0.12 g N+0.2 g P_2O_5+0.1 g K_2O kg^{-1} soil, N2P2K2 = 0.24 g N+0.4 g P_2O_5 +0.2 g K_2O kg^{-1} soil. The N, P_2O_5, and K_2O were applied as urea, superphosphate and potassium sulfate, respectively. One third of the N and all of P and K were applied basally. The remaining N fertilizer was applied on 5 March 2010, before rapid growth of the plants. Each treatment group had ten replicates. In total, 60 pots were established.

Seeds of *P. vulgaris* were collected from Queshan County, Henan Province, P.R.China.

Before sowing, *P. vulgaris* seeds were soaked in 2.5% sodium hypochlorite solution for 1 h. Twenty seeds of approximately the same size were sown in each pot on 10 October 2009. All pots were placed in a rain shelter in randomized positions. All pots were well watered to ensure germination. After one month, the seedlings were thinned to four uniform plants per pot. All pots were measured gravimetrically by weighing and watered with distilled water every other day at 18:00 pm. On 25 March 2010, drought treatments were initiated in half the pots by withholding irrigation; the remaining pots continued to be well watered. Experimental treatments were conducted from 25 March to 15 June (the plants were harvested), 2010.

Growth measurements

At the end of experiment ten plants in each treatment were harvested. Vegetative parts (root, stem and leaf) and reproductive parts (spica) were separated, dried in an oven for 48 h at 70℃ to a constant weight for biomass determination. The dried spicas of *P. vulgaris* were ground into a fine powder and then passed through a 0.5 mm sieve.

Compound analysis

Samples for high performance liquid chromatography (HPLC) determination were prepared using modifications of the methods by Fang et al. (2010). Powdered spica samples (0.10 g) were mixed with 20 ml of 75% methyl alcohol for 30 min and then extracted through 30 min ultrasonic treatment at 20℃ and centrifuged at 10,000 rpm for 10 min. The upper solution was filtered through a 0.45 μm organic membrane filter before injection into the HPLC system.

RA, UA and OA content was determined using an HPLC system consisting of an LC-20AT Liquid Chromatograph (Shimadzu, Kyoto, Japan). The methods for determining three bioactive components have been described previously (Zhang et al., 2007; Wang et al., 2008). Total RA, UA and OA yields were calculated by multiplying the content of RA, UA and OA in the spicas by the dry weight of the spica.

Statistical analysis

The data were subjected to a two-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Data regarding the interactions were reported when the interactions were statistically significant at $p<0.05$. Statistical analyses were conducted using the statistical software package SPSS 11.5 for Windows.

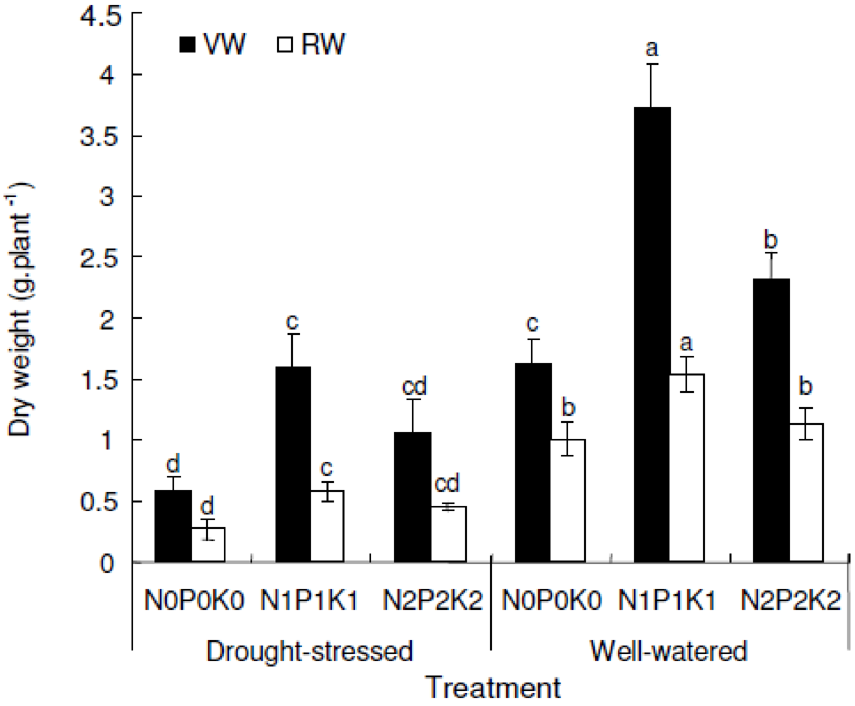

Figure 1. Biomass production of *P.vulgaris.* after various regimens of water and fertilizer. N0P0K0 = without fertilization, N1P1K1 = 0.12 g N+0.2 g P_2O_5+0.1 g K_2O kg^{-1} soil, N2P2K2 = 0.24 g N+0.4 g P_2O_5 +0.2 g K_2O kg^{-1} soil. Nine month-old pot plants of *P. vulgaris.* were analyzed (■)VW vegetative dry weight and (□)RW reproductive dry weight. The letters above the columns indicate significant differences at $P<0.05$ between the watering and fertilization treatments within the same organ (that is, vegetative dry weight or reproductive dry weight).

RESULTS AND DISCUSSION

Effect of fertilization and drought stress on growth

After about nine months of growth, the accumulation of dry matter in both the vegetative and reproductive parts of *P. vulgaris* was found to be significantly affected by the watering and fertilization treatments (Figure 1). The dry weight of the drought-stressed plants (vegetative and reproductive parts) was found to be 58 to 65% lower than that of the well-watered plants when averaged across all fertilizer treatments. Specifically, the dry weight of the reproductive parts (spica) was 73% lower in the unfertilized control, 62% in the N1P1K1 treatment, and 60% in the N2P2K2 treatment compared to plants given the same amounts of fertilizer and plenty of water. Compared to the well-watered plants, the plants under drought stress had a dry weight of vegetative parts that was 64% lower in the unfertilized control, 57% lower in the N1P1K1 treatment, and 54% lower in the N2P2K2 treatment. Under conditions of drought, the plants given fertilizers had more biomass in both the vegetative and reproductive parts than the unfertilized controls. In the drought-stressed plants, maximum biomass production was achieved when appropriate amounts of fertilizer (N1P1K1) were applied: 63 and 53% higher production in vegetative and reproductive parts, respectively, compared to the unfertilized control. Similar results were obtained for plants treated with high fertilizer (N2P2K2): the dry weight of vegetative and reproductive parts was found to increase, by 45 and 40%, respectively, compared to the unfertilized treatment.

These results indicated that *P. vulgaris* growth parameters, including vegetative and reproductive biomass, responded positively to fertilizer application under drought-stress conditions. Wu et al. (2008) pointed out that the negative effect of drought-stress on growth of *Sophora davidii* seedlings could be alleviated with the appropriate fertilizer. Similarly, implementing suitable fertilizers could significantly improve the root and shoot biomass accumulation in *Bupleurum chinense* DC under drought conditions (Zhu et al., 2009). These results implied that providing the proper amount of fertilizer could maintain a relatively high level of growth, enhance the photosynthesis efficiency and improve the efficiency by which other limited resources were used. However, excess fertilizer could severely depress growth or be toxic to plants under drought-stress conditions (Kleiner et al., 1992; Wu et al., 2008). We concluded that *P. vulgaris* could be cultivated in arid and semi-arid regions if it

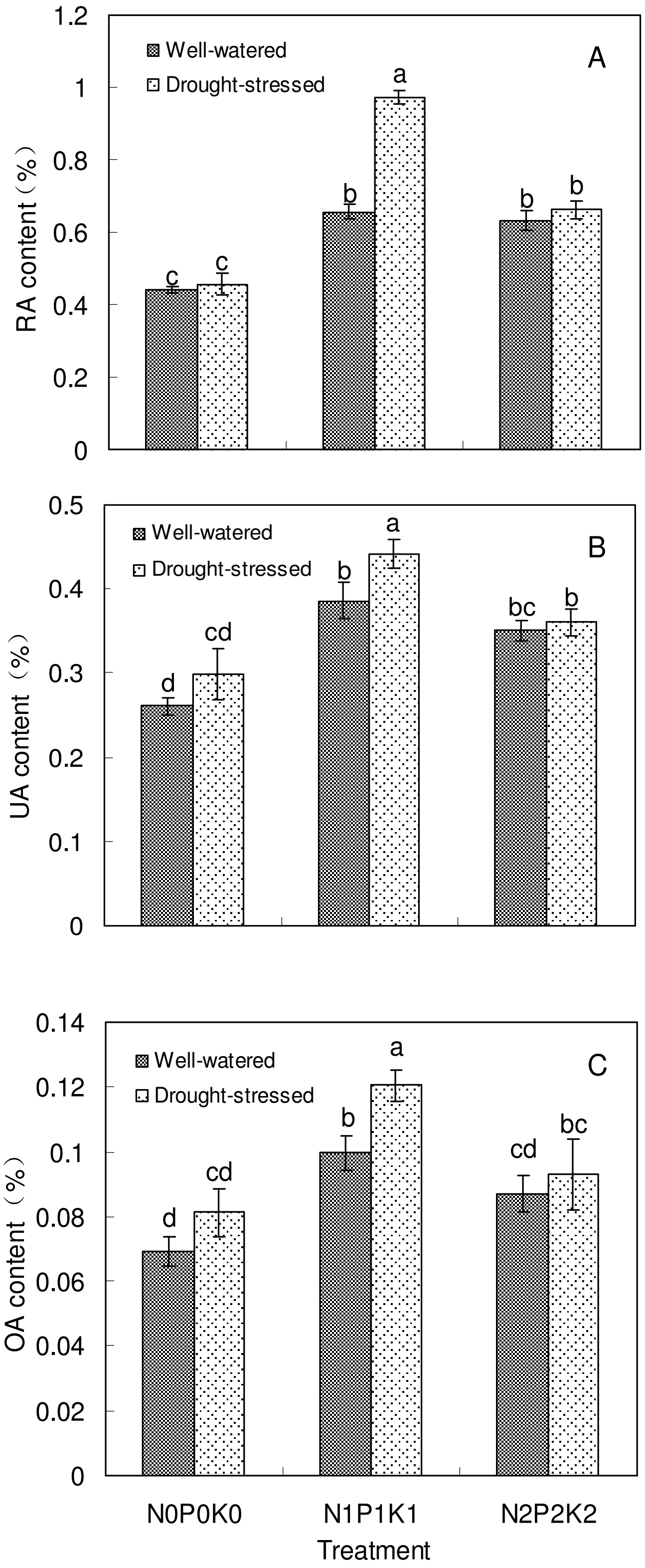

Figure 2. The effect of water and fertilization on RA(A), UA(B), and OA(C) content in P. vulgaris. N0P0K0 = without fertilization, N1P1K1 = 0.12 g N+0.2 g P_2O_5+0.1 g K_2O kg^{-1} soil, N2P2K2 = 0.24 g N+0.4 g P_2O_5 +0.2 g K_2O kg^{-1} soil. The letters above the columns indicate significant differences between the watering and fertilization treatments ($P < 0.05$).

was supplied appropriate amounts of N, P and K fertilizer.

Effect of fertilization and drought stress on compound content

Drought stress increased the RA, UA and OA content of *P. vulgaris* spicas (Figures 2A-C). Under drought conditions, the content of RA, UA and OA in spicas of *P. vulgaris* increased by an average of 14, 9, and 13%, respectively, compared to well-watered plants. These results were consistent with the work of Hura et al. (2009), who reported that the content of phenolic compounds (e.g., ferulic acid) increased in the leaf of triticale seedlings under drought stress. Similarly, drought stress markedly enhanced the total concentrations of monoterpenes and resin acids in the main stem wood of Scots Pine and Norway Spruce Seedlings (Turtola et al., 2003). Drought stress could induce the accumulation of reactive oxygen species (ROS), resulting in oxidative stress in the plant cells, whereas plants could maintain normal metabolism through the antioxidant system for scavenging and detoxifying ROS (Mittler, 2002). In addition, previous studies proposed that oxidative stress played an important role in the formation of secondary metabolites in plants (Kirakosyan et al., 2003). For example, ajmalicine (Jaleel et al., 2007a) and total indole alkaloid (Jaleel et al., 2007b) accumulated in the roots of *C. roseus* under the oxidative stress caused by drought. According to the work of Nacif de Abreu and Mazzafera (2005) on *Hypericum brasiliense* Choisy, the increased production of phenolic compounds and betulinic acid upon exposure to drought and hypoxia may represent an antioxidant response to ROS production. Similarly, terpenoids have been shown to possess antioxidative properties. In particular, volatile isoprenoids were thought to be involved in scavenging ROS and potentially in protecting *Hevea brasiliensis* against oxidative stress (Chen and Cao, 2008). Our previous work has shown that there was an accumulation of oleanolic acid in spicas of *P. vulgaris* in response to mild drought stress. The levels of non-enzymatic antioxidants and the activities of antioxidant enzymes were also enhanced by moderate drought stress (Guo et al., 2009, 2010). Thus, the accumulation of RA, UA and OA in spicas of *P. vulgaris* upon exposure to drought may be an important antioxidant response.

As shown in Figures 2A-C, the RA, UA and OA content in spicas of *P. vulgaris* were found to be significantly affected by the presence of fertilizer. In drought-stressed plants, RA, UA and OA levels were significantly higher with fertilizer treatment (N1P1K1) than in either of the other two treatments (N0P0K0, N2P2K2). When high amounts of fertilizer (N2P2K2) were applied, however, RA, UA and OA levels in spicas of *P. vulgaris* were remarkably lower. These results suggested that excess supply of fertilizers might block RA, UA and OA

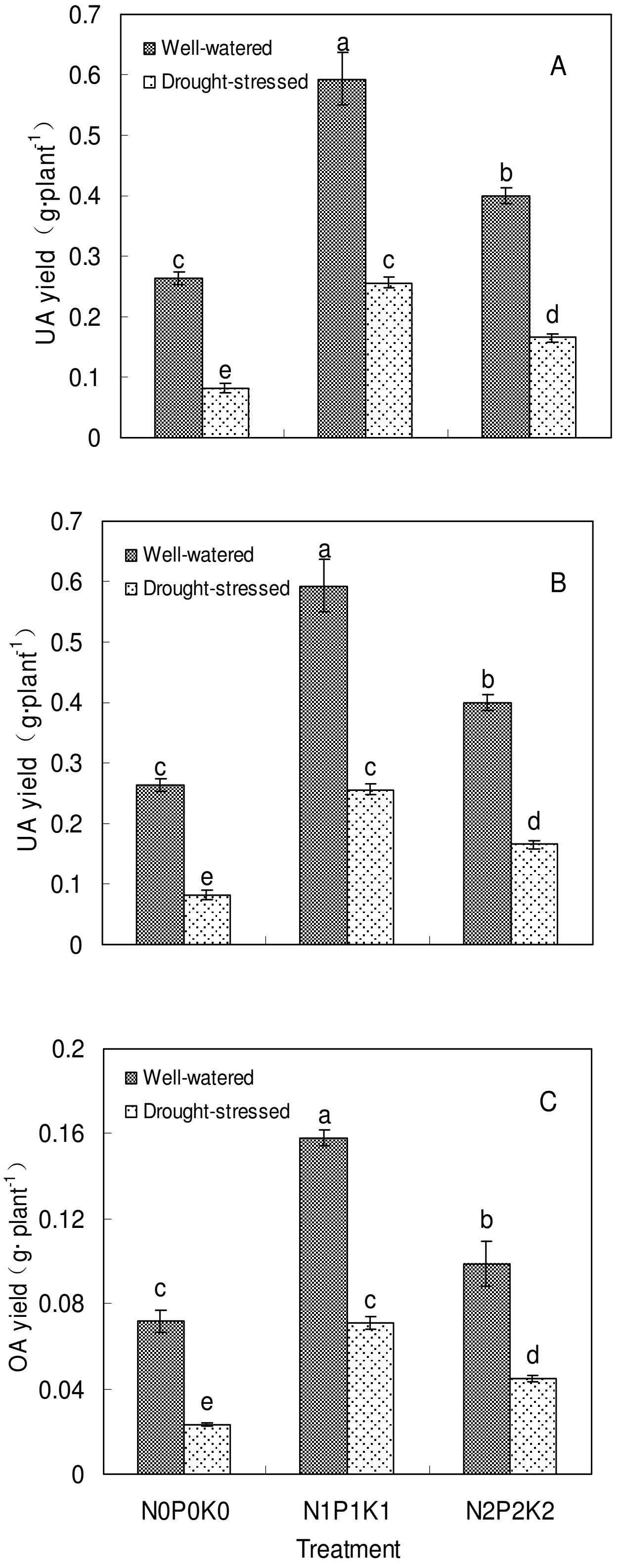

Figure 3. The effect of water and fertilization on RA (A), UA (B), and OA (C) yields in P. vulgaris. N0P0K0= without fertilization, N1P1K1 = 0.12 g N+0.2 g P2O5+0.1 g K2O kg−1 soil, N2P2K2 = 0.24 g N+0.4 g P2O5 +0.2 g K2O kg−1 soil. The letters above the columns indicate significant differences between the watering and fertilization treatments ($P < 0.05$).

biosynthesis.

Effect of fertilization and drought stress on compound yield

The carbon/nutrient balance (CNB) theory (Bryant et al., 1983) and growth-differentiation balance hypothesis (GDBH) (Herms and Mattson, 1992) predicted a trade-off between growth and defense. Because drought stress depressed plant growth, the carbon that was fixed during photosynthesis could be used to form secondary compounds (e.g., phenolics and triterpenes) (Turtola et al., 2003; Hale et al., 2005). Our results indicated that *P. vulgaris* plants in dry conditions decrease vegetative (spica) biomass accumulation by about 65% but also increase RA, UA and OA content in spicas of *P. vulgaris*. Consequently, the total yield of RA, UA and OA decreases by 58, 59, and 61% in drought-stressed plants, respectively, compared to the well-watered plants when averaged across fertilizer treatments (Figures 3A-C). Nevertheless, under drought conditions, total RA, UA and OA yields in the N1P1K1 treatments, when an appropriate amount of fertilizer supplied, were found to be significantly higher than for the other two treatments (N0P0K0, N2P2K2). Specifically, total RA, UA and OA yields, respectively, in the N1P1K1 treatments were 46, 36, and 37% greater than in the N2P2K2 treatment and 78, 68, and 67% greater than in the unfertilized treatment.

Interactive effect of fertilization and drought stress on growth and compound yield

Both water and fertilization were found to significantly influence *P. vulgaris* biomass in the vegetative and reproductive parts (spica), as well as the total RA, UA and OA yields (Table 1). These results indicated that the coordination between water supply and fertilization favors maximal RA, UA and OA yields. Concurrently, it may also suggest that the combined use of fertilizer and properly timed exposure to drought stress would enhance the total RA, UA and OA yields in *P. vulgaris*.

In conclusion, we found that N1P1K1 could improve the ability of *P. vulgaris* to adapt to drought stress by stimulating plant growth and biomass production as well as by increasing RA, UA and OA yields. N2P2K2 showed a negative effect, however.

Thus, in semi-arid and arid regions, an appropriate amount of fertilizer supply would be recommended for the production of *P. vulgaris* but excess fertilizer supply should be avoided.

In the future, more work will be needed to understand how mineral nutrients and drought stress influence the biosynthesis and accumulation of secondary metabolites in *P. vulgaris*.

Table 1. *F* values from ANOVA showing the effects of fertilizer and water on the vegetative and reproductive biomass and the yield and content of RA, UA, OA in *P. vulgaris.*

Variables	Growth parameters		Content			Yield		
	VW	RW	RA	UA	OA	RA	UA	OA
Water (W)	88.59**	106.02**	196.18**	5.85*	24.92**	1781.31**	182.94**	676.14**
Fertilization (F)	33.33**	10.00**	597.35**	29.25**	61.51**	970.21**	61.81**	261.06**
W×F	4.51*	4.20*	126.24**	0.92 ns	2.85 ns	16.12**	5.98*	24.22**

VW, vegetative dry weight; RW, reproductive dry weight; RA, rosmarinic acid; UA, ursolic acid; OA, oleanolic acid; ns, P> 0.05,*P < 0.05, **P < 0.01.

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