ORIGINAL RESEARCH ARTICLE

INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

Economic maximization of alfalfa medically-active phytoconstituents

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Received: March 12, 2016; **Revised:** March 17, 2016; **Accepted**: March 27, 2016

Abstract: Medicinal plants are leading source of phytochemicals. A pot experiment was designed to evaluate the effect of various water regimes (33, 67 and 100% watering) on the ability of alfalfa plants grown in soils with different texture (33, 67 and 100% sand) to synthesize phenolics, non-photosynthetic pigments, vitamins and minerals. Under drought, the amount of total phenols, saponins, tannins, flavonoids and alkaloids increased with the maximum content of total phenols, tannins and flavonoids recorded for plants in soil with 67% sand. For saponins, 33% sand caused the highest values, while alkaloids increased with increasing sand proportion. Water stress also increased the amount of anthocyanins, lycopene and β-carotene. With adequate irrigation, soil with 33% sand caused the highest amount of these pigments while under drought; their values fluctuated among the three soil types. Moreover, the amount of vitamin C, B1 and B2 increased under water deficit; and their amount increased with increasing sand. Furthermore, the amount of sodium, potassium and calcium increased with drought; while the reverse was recorded for magnesium and phosphorus with the maximum titers of all the addressed elements in plants grown in 100% sand. Thence, little water availability may be an efficient technique invigorating alfalfa medicinal efficacy.

Key words: alfalfa, drought, sand, phenolics, non-photosynthetic pigments, vitamins, minerals

Introduction

The use of plants for medicinal purposes dates back thousands of years. Plant-derived pharmaceuticals could significantly contribute to new leads in clinical trials where several phytoconstituents are currently employed in the prevention and treatment of various human diseases [1]. The advantages of plant-based biopharmaceuticals include the low cost of production, purification and storage, the ease of large scale manufacture as well as the high safety when compared with those produced by other routes [2].

One of the foremost medicinal plants is alfalfa (*Medicago sativa* L., family Fabaceae) which has long been used as conventional herbal therapy [3]. Small [4] pointed out to alfalfa as a medicinal plant stating that "perhaps the greatest new potential use of alfalfa lies in the possibility of using it as a biomass crop for the production of pharmaceuticals". Through an exhaustive survey of literatures, alfalfa phytochemicals could be summarized in four categories; namely phenolics, non-photosynthetic pigments, vitamins and minerals. Bioactive phenolics characterized in alfalfa include saponins [5], tannins [6], alkaloids [7] and flavonoids [3]. Medically-active pigments in alfalfa could be classified as anthocyanins, lycopene and ß-carotene [5]. Moreover, alfalfa is a rich source of certain vitamins like vitamin A, B, C, and D [8], as well as minerals like sodium, potassium, calcium, magnesium and phosphorus [9].

Growth, development and consequently the yield of plants are greatly restricted by environmental stressful conditions. In particular, drought is one of the prime abiotic factors adversely influencing plant performance [10]. In addition, high sand proportion in soil may cause marked limitation in plant prosperity because of high porosity favoring the leaching of water and various essential nutrients to the deep layers away from the growing plants. However and in a somewhat unique trend, considerable attention could be paid to utilize such environmental traumas in enhancing plant capability to synthesize certain compounds with medicinal use.

Therefore, the present study seeks not only to ascertain the ability of alfalfa to synthesize various phytochemicals, but it also focuses on exploring how to economically maximize the medical efficiency of that plant. This aim is postulated to be achieved by growing the studied plants under stressful conditions involving various levels of constringent water supply either alone or in combination with elevated sand amounts in the cultivation soil.

Materials and Methods

Plant material and growth conditions

Seeds of alfalfa (*Medicago sativa* L., tolerant cultivar Nubaria 1) were obtained from El-Nubaria, El-Behira Governorate, Egypt. For cultivation, clay and sand were mixed in different ratios to obtain three types of soil with sand proportion of 33, 67 and 100%. Forty-five days post-sowing, the plants growing in each soil type were subdivided into three subgroups so that the plants were subjected to three levels of irrigation; control: irrigating plants when required, moderate drought: withholding 33% of irrigation water and severe drought: withholding 67% of irrigation water. Therefore, the resulting nine treatments could be marked as follows:

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No.	Treatment	Abbreviation
1	33% sand with 100% watering	33% s + 100% w
2	67% sand with 100% watering	67% s + 100% w
3	100% sand with 100% watering	100% s + 100% w
4	33% sand with 67% watering	33% s + 67% w
5	67% sand with 67% watering	67% s + 67% w
6	100% sand with 67% watering	100% s + 67% w
7	33% sand with 33% watering	33% s + 33% w
8	67% sand with 33% watering	67% s + 33% w
9	100% sand with 33% watering	100% s + 33% w

The plants subjected to natural day/night conditions were supplied with the recommended NPK doses and sampled after 75 days from planting.

Quantification of phenolic constituents

According to Malik and Singh [11], total phenols were spectrophotometrically quantified using Folin-Ciocalteau reagent. The mount of saponins, tannins, flavonoids and alkaloids were determined following Obadoni and Ochuko [12], Sadasivam and Manickam [13], Dewanto *et al.,* [14] and Harborne [15]; respectively.

Quantification of non-photosynthetic pigments

Anthocyanins were extracted in acidified methanol followed by centrifugation and the absorbance was measured at 530 and 657 nm [16]. According to Nagata and Yamashita [17], an aliquot volume of the methanolic plant extract was shaken with acetone: hexane mixture then filtered. The absorbance was measured at 453, 505 and 663 nm to calculate the contents of lycopene and β-carotene.

Quantification of vitamins

The amount of vitamin C was estimated as cited from Ogunlesi *et al.,* [18] by titration against 2,6-dichlorophenol indophenol using standard ascorbic acid solution for calibration. To determine vitamin B1, the plant samples were homogenized with ethanolic NaOH solution, filtered, reacted with potassium dichromate solution and measured at 360 nm [19]. For estimation of vitamin B2, plant samples were extracted with ethanol then potassium permanganate and H_2O_2 were added and allowed to stand for 30 minutes over a hot water bath. Na_2SO_4 was then added and the color was measured at 510 nm [19].

Quantification of mineral elements

Plant samples were extracted with HNO_3 and flame spectrophotometry was used to determine sodium and potassium, while calcium and magnesium were measured by atomic absorption spectrophotometry. Phosphorus was quantified following Humphries [20] by digestion using H_2SO_4 followed by adding ammonium molybdate and stannous chloride solutions with shaking then reading the samples at 710 nm.

Statistical analysis

Three replicates were taken for each investigation, and only the mean values were represented. A test for significant differences between means at $P \leq 0.05$ was performed using CoHort/ CoStat software. The treatments were applied to analysis of variance (ANOVA) procedure to determine the least significant difference (LSD) and mean standard error (MSE) so that small letters were denoted, where different letters refer to significant variance with higher degree of variance as the letters are far from each other.

Results

Changes in phenolic compounds

Data in Figure 1 revealed that drought caused significant increase in the amount of total phenols, saponins, tannins, flavonoids and alkaloids. Generally, the increase in the amount of these phenolics was directly proportional to the level of water stress. With respect to the soil, the maximum values of total phenols, tannins and flavonoids were recorded for plants grown in soil with 67% sand. For saponins, soil with 33% sand caused the highest values, while the amount of alkaloids increased with increasing sand.

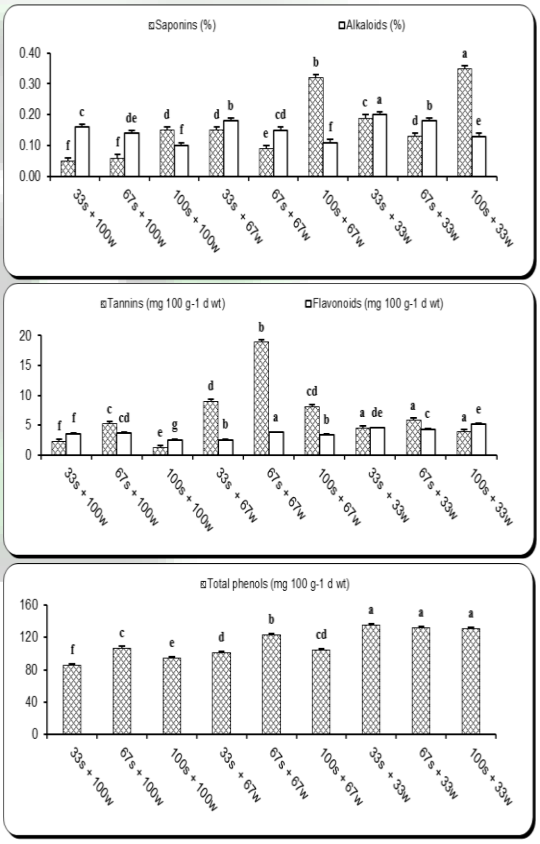

Figure 1: Effect of different water regimes on phenolic content of alfalfa plants grown in different soil types. Vertical bars represent mean standard error with different letters referring to significant difference according to the values of least significant difference at $P \leq 0.05$.

Changes in non-photosynthetic pigments

The results in Figure 2 showed that drought strikingly increased anthocyanins and lycopene amount. The same effect was noticed for β-carotene except for moderate drought which decreased the mount of β-carotene in the plants cultivated in soil with 33% and 67% sand. With adequate irrigation, soil with 33% sand caused the highest amount of the three pigments. Meanwhile, under moderate or severe water stress, the amount of these pigments fluctuated among the three soil types.

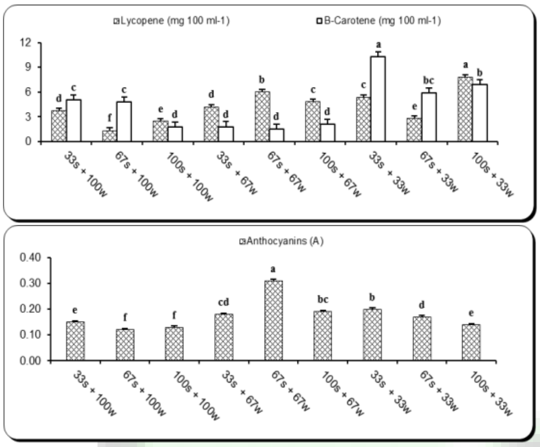

Figure 2: Effect of different water regimes on the amount of non-photosynthetic pigments of alfalfa plants grown in different soil types. Vertical bars represent mean standard error with different letters referring to significant difference according to the values of least significant difference at P ≤ 0.05.

Changes in vitamins

The amount of vitamin C, B1 and B2 markedly increased under water deficit as cleared in Figure 3. The stimulating influence on the amount of vitamin C and B2 was more pronounced for moderate drought than severe stress. Meanwhile, severe stress was more effective than moderate stress in case of vitamin B1. Moreover, the amount of the three vitamins increased with increasing sand proportion in the propagating soil.

Changes in minerals

As shown in Figure 4, the amount of sodium, potassium and calcium remarkably increased with increasing drought level with a decline in the amount of magnesium and phosphorus. The maximum values of all the estimated elements were recorded in plants grown in 100% sand. As an exception, the highest value of phosphorus in water-unstressed and moderately-stressed plants was recorded in soil with 33% sand.

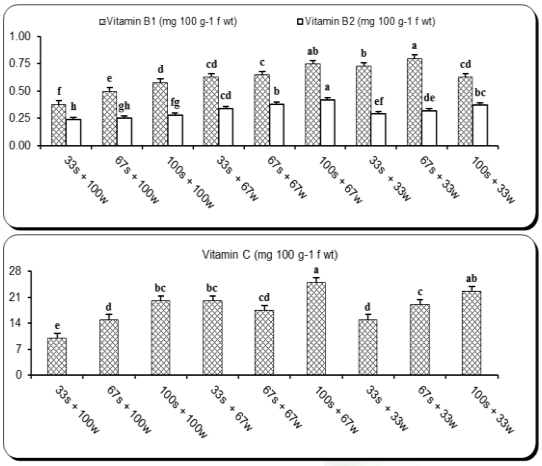

Figure 3: Effect of different water regimes on vitamin content of alfalfa plants grown in different soil types. Vertical bars represent mean standard error with different letters referring to significant difference according to the values of least significant difference at P ≤ 0.05.

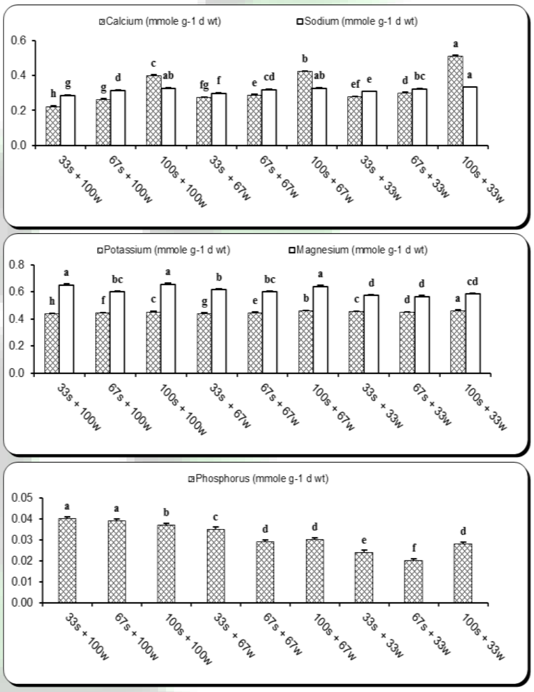

Figure 4: Effect of different water regimes on mineral content of alfalfa plants grown in different soil types. Vertical bars represent mean standard error with different letters referring to significant difference according to the values of least significant difference at P ≤ 0.05.

Discussion

The potent use of a plant for health care may rely on its efficiency to synthesize certain metabolites. Thus, it is of great importance not only to prove certain plant potentiality to produce some medically-active phytochemicals but also to maximize such capacity. Plant phenolics have drawn great interest due to their efficient antioxidant characteristics and their significant effects in preventing various oxidative stress-related diseases [21]. In the present study, water deficit enhanced the synthesis of total phenols, saponins, tannins, flavonoids and alkaloids. In accordance with these results, a significant increase in total phenol concentration was reported within seven days of water shortage in clover plants [22].

Phenolics are stress-induced metabolites that accumulate in plant tissues as a result of different stress stimuli mainly to participate in reactive oxygen species (ROS) scavenging [23]. The observed increase in alfalfa content of total phenols could be attributed to the enhanced activity of phenylalanine ammonia lyase, a key enzyme involved in the anabolism of phenols. In this connection, Lee *et al.,* [22] reported a similar positively correlated increase in phenylalanine ammonia lyase activity and total phenolics in response to stress.

Plant phenolics include saponins, tannins, alkaloids and flavonoids [21]. Plant saponins are a group of naturally-occurring triterpene or steroid glycosides with a huge number of pharmacologically-active compounds [24]. In consistence with our results that indicate an obvious overproduction of saponins in stressed alfalfa plants, saponin content of guayule plants increased by water deficit [25].

Plant tannins are a large group of polyphenolics found in several plant species with protective function in the bark of roots, stems or any outer layer of the plant [26]. More interestingly, plant tannins were reported to be associated with many human physiological activities [27]. In agreement with the recorded rise in tannins content of water-stressed alfalfa plants in the present study, increased tannin content was documented by Osuagwu *et al.,* [28] in *Ocimum gratissimum* and *Gongronema latifolium* plants.

Flavonoids are one of the largest classes of plant phenolics performing different functions in plant system, including pigmentation and defense. Flavonoids were further reported to have multiple medicinal properties [29]. Coinciding with the results obtained herein, Haghighi *et al.,* [30] recorded that water stress enhanced the accumulation of flavonoids in *Plantago ovata* plants. Moreover, they postulated that this increment in flavonoid content might be due to the induction in enzymatic activity occurring under stress.

Alkaloids are considered by Kar [31] to "exhibit a wide-spectrum and complete diversity of complex structures responsible for their extra-ordinary pharmacological activities". Studies on plant alkaloid composition cleared that stress conditions might exert paramount influence on the individual alkaloids of such plants as adaptive mechanism to stress [32]. Accumulation of alkaloids in response to stress was also recorded in *Catharanthus roseus* shoots [33].

Among the plant metabolites recognized with powerful pharmaceutical activities, most non-photosynthetic pigments were documented to have health care attributes [34]. Examples of theses medically-active pigments are anthocyanins that possess antioxidant and antiinflammatory activities [35]. In addition, carotenoids have been shown to possess possible health benefits [36]. Lycopene is one of the major carotenoids known for its potentiality to prevent cancer as well as cardiovascular diseases. Another carotenoid is β-carotene with potent medical activities [37].

In the present study, water stress generally increased the amount of anthocyanins, lycopene and β-carotene in alfalfa plants. With respect to anthocyanins, plant tissues with high anthocyanin titers are usually rather resistant to drought [38]. Anthocyanins may modulate plant tolerance to drought by stabilizing water potential as compatible solutes, maintaining redox hemostasis of cellular fluids and mitigating the ill impact of ROS [38].

Under water shortage conditions, accumulated quantities of carotenoids were also reported in other studies [39]. For instance, the results obtained by Sedghi *et al.,* [40] showed that the concentration of carotenoids; namely lycopene and β-carotene, increased under water deficit in *Calendula officinalis* plants. The mechanism behind carotenoids-induced drought tolerance may lay in being the substrates for the biosynthesis of some plant growth regulators especially stress-ameliorative ABA. Furthermore, carotenoids may play a pivotal role as non-enzymatic antioxidants by scavenging ROS particularly singlet oxygen [41].

The synthetic and degrading pathways as well as the functions of vitamins have been extensively demonstrated. In the present investigation, the amount of vitamin C, B1 and B2 in the studied alfalfa plants significantly increased when the plants were subjected to water stress. In agreement with this finding, Rapala-Kozik *et al.,* [42] and Krauss *et al.,* [43] recorded that water stress led to increased thiamine content in maize plants and enhanced vitamin C in tomato; respectively.

The significance of vitamins as plant nutrients and more importantly as stress counteracting agents has been verified. The concentration of vitamins as antioxidants was intensively reported to be affected by water stress [44]. In plants, considerable attention has been given to the non-enzymatic antioxidant defense system because of its central role in protecting the chloroplasts, mitochondria and other cellular compartments from oxidative damage.

Minerals are essential nutrients for every living cell as they assist in the body functions and help in the assimilation of vitamins along with other nutrients. The concentration of mineral elements in plants is usually fluctuated under various environmental circumstances especially water stress [44]. In the present study, water depletion brought about marked increase in sodium, potassium and calcium content, while it decreased the amount of magnesium and phosphorus in alfalfa plants. Matching with our results, water stress could result in increased levels of sodium [45], potassium and calcium [46] in various plant species. On the other hand, water stress caused significant reduction in magnesium [47] and phosphorus [48] content of different plants.

The recorded increase in sodium, potassium and calcium content in response to stress, either drought or high sand proportion, may be an adaptive feature of a tolerant variety (*Medicago sativa* L. cultivar Nubaria 1) in a trial to increase the cellular osmotic pressure that would help to maintain more water. On the other hand, the lower amount of magnesium and phosphorus in alfalfa plants could be regarded as a negative consequence of stress that may cause depletion or less absorption of these two elements.

Therefore, it could be concluded that exposing alfalfa plants to water deficit could enhance its ability to over-produce some medically-active phytochemicals. Stress-induced accumulation of these metabolites might be one of the strategies employed by a stress-tolerant cultivar of alfalfa to resist the ill impact of unsuitable growth conditions employed by irrigation water withholding combined with raising sand proportion in the cultivation soil. Economically, such a strategy to force a plant to enhance its phytochemical profile would be efficient as it is easy, low cost, low risk and applicable technique.

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Cite this article as:
Mickky B.M., Abbas M.A., and El-Shhaby O.A. Economic maximization of alfalfa medically-active phytoconstituents. *International Journal of Bioassays* 5.4 (2016): 4984-4990.

Source of support: Nil
Conflict of interest: None Declared