RATE AND FORMS OF MINERAL NUTRITION CAN INFLUENCE DRY MATTER ACCUMULATION AND SAPONIN CONTENT OF PUNCTURE VINE (TRIBULUS TERRESTRIS L.)

G.I. Georgiev¹, A. Ivanova², P. Mechkarova², A. Ivanova¹, L. Popova¹
¹Institute of Plant Physiology, BAS, Sofia, Bulgaria
²Institute of Organic Chemistry with Centre of Phytochemistry, BAS, Sofia, Bulgaria
Correspondence to: Georgi I. Georgiev
E-mail: gig@bio21.bas.bg

ABSTRACT
Effect of mineral nutrition (soil or foliar supply of nutrients) on the growth, dry matter and saponin content of the shoot biomass of Bulgarian variety puncture vine (Tribulus terrestris L.) grown on soil as pot experiment in greenhouse were studied. Soil fertilization rate of 100mgN/kg or 90P/kg of dry soil, oppositely to the results obtained from the foliar fed plants (0.3 % solution of liquid fertilizer Agroleaf⁶ (Scotts Co, USA) with formulation N₁₂P₅K₅, increased shoot total N and P without significant change of dry matter. Changes of total reducing sugars, amino acids, phenolics and flavonoids and activity of leaf photosynthetic apparatus (chlorophyll (Chl) a, b and carotenoids content and parameters of chlorophyll a prompt fluorescence) were found to relate to the variation of individual saponin content analysed by HPLC technique. Soil fertilized plants in contrast to the foliar fed plants showed more protodioscin, prototribestan and dioscin than control but contained less of flavonoid glycoside rutin.

Keywords: puncturevine (Tribulus terrestris L.), mineral nutrition, saponins, growth

Abbreviations used: NPK- nitrogen, phosphorus, potassium, HPLC- high performance liquid chromatography

Introduction
Puncture vine (Tribulus terrestris L., Zygophyllaceae) is a medicinal plant widely used as a drug supplier in pharmaceutical industry (2, 9). Its biomass is known as a source of steroidal saponins mainly from the furostanol and spirostanol types. Extracts of plants have been used for years in traditional or modern medicine as food supplements with substantial therapeutic or health effects on humans. Some years ago it was found that plant species with Bulgarian origin contain more saponin glucosides or sulfoderivatives of saponins from the furostanol or spirostanol structural groups in comparison with plants grown in other countries belonging to Maritime climates (1,4,9). Irrespective to the number of investigations concerning botanic, ecological, geographical or chemo type of this plant species little is known of the relationship between growth and accumulation of saponins in its biomass. However, due to extensive search of plant material for commercial purposes from different pharmaceutical companies more or less natural habitats of plant are considered as exhausted. To solve this problem it will be necessary to develop efficient methods for cultivation of this plant on arable lands. However, this approach needs substantial knowledge on physiology and biochemistry of the cropping method. Mineral nutrition is powerful and important tool for plant growth and productivity improvement (3,7,8). There have been several attempts to cultivate this species on arable lands but no adequate response has been obtained (3). One important question of cultivated herbs is how to increase their productivity without lost of quality of the product. Steroidal saponins of puncture vine species belong to the group of secondary metabolites of the terpenoid pathway (4). Terpenoids are synthesized in the acetate-mevalonate as in microorganisms or in non-mevalonate pathway in plants, however, little is known about their regulation and manipulation. There are several hypotheses suggesting how to regulate relationships between primary and secondary metabolism but no clear evidences have been obtained yet (7,5,6). It is believed that regulation of amino acid metabolism can be a key factor for turning on the secondary metabolism in plants especially under some growth constrains such as nitrogen deficiency or excess (6).

Regarding these considerations we have attempted to test how different forms or rate of nitrogen or phosphorus nutrition will influence the growth, dry matter and saponin content of plants grown in soil pot experiments. Accordingly,
some physiological and biochemical parameters of the ratio between primary and secondary metabolism have been studied as well.

**Materials and Methods**

Puncture vine plants (*Tribulus terrestris* L.) were grown under naturally lit and heated green house (day/night temperature – 32/19°C) from May to September. Ripen burrs were used for germination of plants. They were collected from the plants grown in natural habitat from the Pazardjick region, in Bulgaria. 12 randomly selected seeds were presowed in the clear moist quartz sand for 3 days and then transferred to the earthen pots containing 4 kg of dry leached cinnamon meadow soil also known as Chromic Luvisols (FAO) for further growth. 4 plants per pot were left after successful germination. Soil moisture in pots was kept at 70 % of full moisture capacity during the entire period of the experiment. In one set of pots, 4 weeks after the germination, the plants (budding stage of growth) were sprayed with commercial product of 0.3% solution of liquid fertilizer (Agroleaf®, Scotts co, Ohio,USA) with the formulation N:P:K$_5$ + microelements. In another set of pots soil was amended with NH$_4$NO$_3$ to reach final concentration of soil N (soil + fertilizer N) – 100 mgN/kg soil. Another set of pots was accommodated for growing plants under higher soil P fertilization. KH$_2$PO$_4$ was applied to 90 mgP/kg soil as final concentration (soil contains total 25 mg P/kg). Aerial parts of puncture vine plants were collected after 4 weeks of growth for analysis. 5 plants were used for determination of fresh/dry matter and phenologic observations. Aliquots of dried material were used for analysis for free amino acids (13), reducing sugars (2) and total phenols (10) and flavonoids (15). Elemental analysis of NPK in plant shoot was done according to methods described in details elsewhere (11). Leaf pigment content and chlorophyll a fluorescence parameters were also assayed as described in (5). Extraction, isolation and HPLC assay of the saponins from the aerial parts of plants were carried out (1). Statistical analysis of the means (3-6 replicates per measurement) was performed by program Statgraphics plus (Statgraf Co,USA).

**Analytical HPLC method**

An HPLC system La Chrom Elite consisting of L-2130 pump equipped with gradient controller and UV detector L-2400 was used. The separation was performed on 250 x 4.6 mm i. d., 5 µm, Inertsil ODS-2 column (Tokyo, Japan) with MetaGuard Pursuit direct connect guard column from Varian was used for all separations. The mobile phase which consisted of phosphoric acid buffer with pH-3 (A) and acetonitrile (B) was used for gradient elution. The flow rate was adjusted to 1.0 ml/min, the detection wavelength was at 203 nm. All separations were performed at ambient temperature.

**Sample preparation**

The finely powdered plant material (1g, leaves and fruits, 1 : 1) was extracted three times with 5.0 ml of 50% aqueous acetonitrile by sonication for 15 min. The extracts were combined, after filtration in 20.00 ml volumetric flask and the volume was adjusted to 20 ml with the solvent use for extraction. Prior injection, all samples were filtered through a 0.45-µm Chromafil 0-45/25 Machery-Nagel. Every sample solution was injected in triplicate with injection volume of 25µl.

**Results and Discussion**

Foliar fed plants 10 days after the treatment showed slightly inhibited growth (Table 1). Decreased total biomass related to the inhibited plant development (Table 1). In contrast, soil fertilized plants with 100 mgN/kg soil or 90 mgP/kg soil had improved growth and accumulated more dry matter (214 % of control).

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Average length of stems, cm</th>
<th>Number of stems per plant</th>
<th>Number of leaves per plant</th>
<th>Number of flowers per plant</th>
<th>Number of burrs per plant</th>
<th>Fresh matter, g/plant</th>
<th>Dry matter, g/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foliar fertilized plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.12a</td>
<td>3.25a</td>
<td>27.25a</td>
<td>3.75a</td>
<td>10.52a</td>
<td>1.26</td>
<td>0.39a</td>
</tr>
<tr>
<td>0.3% Agroleaf</td>
<td>13.12ab</td>
<td>3.00a</td>
<td>27.54a</td>
<td>1.25b</td>
<td>6.25b</td>
<td>1.20</td>
<td>0.36ab</td>
</tr>
<tr>
<td>Soil fertilized plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.45a</td>
<td>4.3a</td>
<td>30.5a</td>
<td>4.2a</td>
<td>12.13a</td>
<td>0.54a</td>
<td>0.14a</td>
</tr>
<tr>
<td>100 mg N/kg</td>
<td>18.54b</td>
<td>4.9ab</td>
<td>34.7b</td>
<td>4.1a</td>
<td>13.80b</td>
<td>1.02b</td>
<td>0.24b</td>
</tr>
<tr>
<td>90 mg P/kg</td>
<td>17.43c</td>
<td>5.1c</td>
<td>32.7c</td>
<td>4.0a</td>
<td>14.90c</td>
<td>1.41c</td>
<td>0.29c</td>
</tr>
</tbody>
</table>

Data are means of 6 replicates. Means with equal letters are not significantly different, t-test (P≤0.05)
These changes of plant productivity were related to some physiological and biochemical indexes of plants (Table 2). Leaf feeding with nitrogen decreased free amino acid content of shoot (91.7% of control) but increased the content of reducing sugars (130.3% of control). The changes of basic metabolites of primary metabolism were accompanied with increased concentrations of NPK of the leaves. Although these metabolites in soil fertilized plants were increased phenolics as representatives of secondary metabolites of plants were negatively affected. Foliar fed plants had also less flavonoids in the shoots.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total free amino acids, mM/g DW</th>
<th>Total reducing sugars, mg/g DW</th>
<th>Total soluble phenols, mg/g DW</th>
<th>Total soluble flavonoids, µg/gDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.88a</td>
<td>48.55a</td>
<td>2.85a</td>
<td>10.02a</td>
</tr>
<tr>
<td>0.3% Agroleaf</td>
<td>4.52b</td>
<td>63.27b</td>
<td>2.36b</td>
<td>8.30ab</td>
</tr>
<tr>
<td>Soil fertilized plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.06a</td>
<td>81.15a</td>
<td>3.77a</td>
<td>11.24a</td>
</tr>
<tr>
<td>100 mg N/kg</td>
<td>5.79b</td>
<td>71.26b</td>
<td>3.88a</td>
<td>10.89ab</td>
</tr>
<tr>
<td>90 mg P/kg</td>
<td>5.17ab</td>
<td>55.41c</td>
<td>3.69a</td>
<td>11.37ab</td>
</tr>
</tbody>
</table>

Data are means of 3 replicates. Means with equal letters are not significantly different, t-test (P≤0.05)

The content of protodioscin was highest in leaves under this treatment. Analysis of results indicated that the source and rate of mineral nutrition of puncture vine could be an important tool for regulation of plant productivity and saponin content of puncture vine grown under cultivation conditions. The presented results support evidence that the contradiction between primary and secondary metabolism can be overcome by optimizing growth conditions of plants (6, 11). However, the significance of these results for Tribulus terrestris growth should be augmented and clarified in field trial experiments.

**TABLE 3**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Chl. a</th>
<th>Chl b</th>
<th>A+b</th>
<th>Carotenoids</th>
<th>Chlorophyll fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F/Fo</td>
</tr>
<tr>
<td>Foliar fertilized plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control leaf</td>
<td>1.557a</td>
<td>0.451a</td>
<td>2.009a</td>
<td>0.294a</td>
<td>3.945a</td>
</tr>
<tr>
<td>0.3% Agroleaf</td>
<td>1.585ab</td>
<td>0.434b</td>
<td>2.019b</td>
<td>0.312b</td>
<td>3.770b</td>
</tr>
</tbody>
</table>

Data are means of 3-5 replicates. Means with equal letters are not significantly different, t-test (P≤0.05)
Main saponins and flavonoid of shoots from puncture vine (*Tribulus terrestris* L.) plants fertilized by leaves or soil with excess of P

<table>
<thead>
<tr>
<th>HPLC analysis of main components of plant extracts, mg/g DW</th>
<th>Control of foliar fed plants</th>
<th>Foliar fed plants 0.3% Agroleaf N\textsubscript{12}P\textsubscript{5}K\textsubscript{5}</th>
<th>Control of soil fertilized plants with 90mgP/kg</th>
<th>Soil fertilized plants with 90mgP/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>2.98a</td>
<td>2.50a (83.9)</td>
<td>3.44a</td>
<td>2.23a (64.8)</td>
</tr>
<tr>
<td>Protodioscin</td>
<td>11.25b</td>
<td>13.50b (120.0)</td>
<td>8.29b</td>
<td>11.23b (135.4)</td>
</tr>
<tr>
<td>Prototribestin</td>
<td>16.35c</td>
<td>9.33c (57.0)</td>
<td>5.82c</td>
<td>8.05c (138.3)</td>
</tr>
<tr>
<td>Dioscin</td>
<td>1.74d</td>
<td>1.30d (74.7)</td>
<td>1.37d</td>
<td>1.59d (116.0)</td>
</tr>
</tbody>
</table>

Data are means of 3 replicates. Means with equal letters are not significantly different, t-test (P≤0.05) ; Values in parenthesis represent % from control.

Content of steroidal saponins and rutin in *Tribulus terrestris* fertilized by leaves or soil with excess of P

<table>
<thead>
<tr>
<th>Control of foliar fed plants</th>
<th>Foliar fed plants 0.3% Agroleaf N\textsubscript{12}P\textsubscript{5}K\textsubscript{5}</th>
<th>Control of soil fertilized plants with 90mgP/kg</th>
<th>Soil fertilized plants with 90mgP/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/g DW</td>
<td>%</td>
<td>mg/g DW</td>
<td>%</td>
</tr>
<tr>
<td>Rutin</td>
<td>2.98±0.19</td>
<td>0.3</td>
<td>2.50±0.05</td>
</tr>
<tr>
<td>Protodioscin</td>
<td>11.25±0.51</td>
<td>1.1</td>
<td>13.50±0.19</td>
</tr>
<tr>
<td>Prototribestin</td>
<td>16.36±0.39</td>
<td>1.6</td>
<td>9.33±0.19</td>
</tr>
<tr>
<td>Dioscin</td>
<td>1.74±0.02</td>
<td>0.2</td>
<td>1.30±0.02</td>
</tr>
</tbody>
</table>

**Conclusions**

1. Soil fertilization of *Tribulus terrestris* with 100mgN/kg soil or 90 mgP/kg in pot experiment significantly increased dry matter and saponin content of shoots.

2. Growth of foliar fed plants did not respond adequately to the applied nutrients but saponin content of aerial mass was partially increased.

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**REFERENCES**


