PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF Achillea millefolium GROUP-RELATED CULTIVAR PROA TO DIFFERENT NITROGEN REGIMES

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ABSTRACT
In the present study the effect of different nitrogen amounts (0 g [N₀], 0.45 g [N₁], 0.9 g [N₂] and 1.35 g N [N₃] per pot, corresponding to 0 kg·ha⁻¹, 50 kg·ha⁻¹, 100 kg·ha⁻¹ and 150 kg·ha⁻¹ N application in field conditions) and application times (at sowing, vegetative stage and pre-bloom stage) on the yield, relative water content, total nitrogen content and nitrogen use efficiency in plants of the Achillea millefolium group-related cv. Proa was investigated. Antioxidant activity, flavonoid content, rate and composition of the essential oils were also determined. Application of N₁ increased the dry herbage and flos production, total nitrogen amount and nitrogen use efficiency, without affecting the essential oil rate while reducing the antioxidant activity. Higher amounts (N₃) of nitrogen maintained the dry herbage and flos yield at levels similar to N₁, but decreased the antioxidant activity and rate of the essential oil. The major component of the essential oil, chamazulene, was negatively (N₂, N₃) or not influenced (N₁) by the nitrogen application, while variable changes were registered in the content of the other components. The data point to the nitrogen regime being a subtle tool for regulating the performance of cv. Proa plants.


Keywords: Achillea millefolium group, cv. Proa, nitrogen application, dry matter, essential oil, antioxidants, chamazulene

Abbreviations: GC–MS: Gas Chromatography–Mass Spectrometry; PFD: photon flux density; RWC: relative water content

Introduction
During the last decades the production volume of the medicinal plants has been constantly increasing as a result of the rising demand for natural products and introduction of their new uses in the pharmacy, cosmetics and food industries (5). Plants of the genus Achillea L. (Asteraceae family), referred to as “yarrow”, have been known for over 3000 years (23), and have been used in folk medicine against several ailments such as skin inflammations, spasmodic and gastrointestinal disorders, hepatobiliary complaints (7). The medicinal properties of Achillea are appreciated worldwide and the plants are included in the national Pharmacopoeias of some countries like Germany, the Czech Republic and France (9).

Studies on Achillea millefolium s.l. (sensu lato) have identified different components such as essential oils, sesquiterpenes and phenolic derivatives constituting a group of secondary metabolites with powerful pharmacological activity (7). One of the main constituents of the essential oil, chamazulene, contributes to the anti-inflammatory efficiency by antioxidant-type inhibitory interactions (28). On the other hand, phenolic compounds – flavonoid and phenylcarboxonic acid complexes – are mainly recognized as responsible for the anti-inflammatory (9), anti-microbial (1), choleretic (6) and cytotoxic (31) activities of the plant. One of the most important types of phenolic compounds are flavonoids and their amount is defined as linked to the antioxidant activity of plant extracts (17). Antioxidants are accountable to prevent oxidation reactions which are the major causes for cellular deterioration (33).

Medicinal plants are generally collected from the wild flora, which makes the quality of their extracts unpredictable due to the effect of various environmental factors (12). Appropriate management of growth conditions may be effective in increasing the yield and quality potential of the plants (24). One of the most important environmental factors, nutrient deficiencies, may affect the plant growth, increase the production of reactive oxygen species (ROS) and enhance the antioxidant activity displayed by phenolic derivatives (18). Thus, in nitrogen starvation conditions accumulation of phenolic compounds such as flavonols (32), anthocyanins (10) and phenolic acids (19) was established. Accordingly, a decrease in the amounts of total nitrogen, amino acids, chlorophyll and carotenoids is reported for the nitrogen deficient-plants of Achillea collina Becker ex Rchb., whereas the amount of phenolics and antioxidant capacity are higher in leaves and roots (14, 15). Furthermore, improved nitrogen nutrition leads to higher values for leaf size, number of flowering stalks (27), fresh and dry weight of shoots (15, 25).

Therefore, the purpose of this study was to evaluate the effect of different nitrogen amounts and times of application...
on the yield, relative water content, total nitrogen and nitrogen use efficiency in plants of the *Achillea millefolium* group-related cv. Proa. Antioxidant activity, flavonoid content, rate and composition of the essential oil were also investigated.

Materials and Methods

Plants and growth conditions

Plants of the cv. Proa (related to *Achillea millefolium* group) were used as experimental material. Seeds of cv. Proa were supplied from Pharmasaat GmbH (Germany). Pot experiments were conducted in 2009, in a fully controlled climate chamber. The pots were 60 cm in height and 17 cm in width and were supplied with a net at the bottom to maintain infiltration. Each pot had 15 kg soil substrate and contained one plant. Sterilized field soil of the clay-loamy type (2.2 % organic matter, pH 7.6) was used as a substrate. Seeds were germinated and 7-week-old seedlings were transferred to pots. Plants were grown at 20 °C to 25 °C and 40 % to 50 % air humidity during the whole growth period. The average photon flux density (PFD) was approximately 400 µmol·m⁻²·s⁻¹ (12 h light/12 h dark).

Treatments

The study was carried out in a randomized experimental design with two factors and three replicates. The first factor was the nitrogen amount (ammonium nitrate), namely 0 g (N₀), 0.45 g (N₁), 0.9 g (N₂) and 1.35 g (N₃) per pot, corresponding to 0 kg·ha⁻¹, 50 kg·ha⁻¹, 100 kg·ha⁻¹ and 150 kg·ha⁻¹ N application in field conditions. The second factor was the time of nitrogen fertilization performed at three different points during the growth cycle: at sowing (A₁), at the vegetative stage (A₂), and the pre-bloom stage (A₃).

Sampling

All analyses were done at the end of the experiment when plants were fully bloomed. The whole plants (referred to as herba) were divided into flowers (flos) and stems and leaves (bottom herbage), which were separately analyzed.

Dry weight and relative water content (RWC)

Dry weight was determined after drying at 35 ºC. RWC was calculated based on the dry and fresh weight values according to the following formula:

$$RWC = \frac{(FW - DW)(S - DW)}{S - DW},$$

where $RWC$ is relative water content, $FW$ is fresh weight, $DW$ is dry weight, and $S$ is saturated weight of leaves.

Nitrogen content and nitrogen use efficiency

Finely ground dry plant samples were used for measuring the nitrogen content by the standard macro-Kjeldahl method (11). Nitrogen use efficiency was calculated based on the ratio of total biomass production of plants and total nitrogen uptake from the soil.

Rate and composition of essential oil

The air-dried flos samples were submitted for 3 h to stem distillation using a Likens–Nickerson type apparatus to produce the essential oil. The components of essential oil were analyzed by GC–MS (Hewlett Packard, USA) equipped with an HP 5973 mass selective detector. The capillary column was HP-5MS UI, Agilent Technologies, USA (60 m × 0.25 mm × 0.25 µm).

Antioxidant activity

Antioxidant activity (FRAP assay) was determined according to the method of Benzie and Strain (8). Plant sample (0.5 g) was ground with 20 mL 80 % (v/v) ethanol and then centrifuged for 20 min at 4 °C. The reaction was started by adding 0.05 mL extract to 1.50 mL FRAP reagent and 0.15 mL distilled water. The absorbance of the reaction mixture was measured spectrophotometrically (Cary 50®) at 593 nm. The results were expressed as µmoles FRAP in 1 g dry matter.

Flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay (31): 0.3 mL of 5 % NaNO₂ was added to 0.3 mL extract. After 5 min 0.3 mL of 10 % AlCl₃ solution was added. Then, after 6 min, 2 mL of 1 mol·L⁻¹ NaOH was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well and kept in darkness for 30 min. The absorbance was measured spectrophotometrically (Cary 50®) at 510 nm. Total flavonoid content of plant samples was expressed as mg rutin in 1 g dry matter.

Statistical analysis

Data were subjected to analysis of variance for each parameter. All data were analyzed by computer software (standard ANOVA analysis). The means were compared by using the LSD test described by Steel and Torrie (30).

Results and Discussion

Dry matter yield

The effects of different nitrogen amounts and application times on dry herbage, dry flos and dry bottom herbage of cv. Proa are demonstrated in Fig. 1. The highest dry herbage yield (15.6 g/plant) was obtained from N₃ treatment, whereas the lowest values were recorded in N₀ (13.2 g/plant) and N₁ treatments (13.6 g/plant). The dry flos weight was higher in N₂ (3.4 g/plant) and N₃ (3.2 g/plant) than in N₁ (1.7 g/plant) and N₀ treatments (2.4 g/plant). An increase in the dry herbage yield as a result of increased nitrogen amounts in the soil was reported for *Origanum vulgare* L (3) as well as for *Achillea collina* Becker ex Rchb. (15). However, plants like *Artemisia annual* L. showed no better yield potential with additional rates of nitrogen (27). The effect of the different times of application of the nitrogen fertilizer on the dry herbage yield of cv. Proa was not statistically significant (Fig. 1). The effect of higher nitrogen concentrations on the yield formation was better marked for dry flos than for the lower parts of the plants (dry bottom herbage) (Fig. 1). Hence, it could be suggested that the additional nitrogen supply could improve the yield of flowers, the economically most important parts of the plant.
the vegetative stage (A3: at the pre-bloom stage) on nitrogen content of dry herbage weight of cv. Proa plants. Mean values marked by the same letters are not significantly different at the 0.05 level, using the LSD test.

Fig. 1. Effects of different nitrogen amounts (0 g [N0], 0.45 g [N1], 0.9 g [N2], and 1.35 g [N3] per pot) and application times (A1: at sowing, A2: at the vegetative stage, A3: at the pre-bloom stage) on dry herbage, dry flos and dry bottom herbage weight of cv. Proa plants. Mean values marked by the same letters are not significantly different at the 0.05 level, using the LSD test.

Fig. 2. Effects of different nitrogen amounts (0 g [N0], 0.45 g [N1], 0.9 g [N2], and 1.35 g [N3] per pot) and application times (A1: at sowing, A2: at the vegetative stage, A3: at the pre-bloom stage) on nitrogen content of dry herbage and nitrogen use efficiency of cv. Proa plants. Mean values marked by the same letters are not significantly different at the 0.05 level, using the LSD test.

RWC

RWC of plants varied between 35.3 % and 39.9 % as a result of nitrogen levels, the difference between treatments being statistically non-significant. The different times of application also had no statistically significant affects on RWC (data not shown). Bahavar et al. (4) reported that the water content of plant tissues rose with an increase in the nitrogen amount in the soil. However, there are also findings that RWC is not affected by additional nitrogen amounts (29), which is compatible with our results on cv. Proa.

Total nitrogen content and nitrogen use efficiency

The availability of nitrogen is the primary, considerable factor limiting plant growth in many environments; therefore reactions of plants to different nitrogen amounts are subject of significant interest. The responses of various medicinal plants, such as Melissa officinalis L. (16), Thymus vulgaris L. (13), and Lavandula angustifolia Mill. (2), to nitrogen fertilization have been reported. In the present study the highest total nitrogen content per plant (345.6 mg/plant) was found in the N1 treatment, whereas the lowest one (265.6 mg/plant), in the N0 treatment (Fig. 2). Although an increase in the total nitrogen content of plants was observed with increasing the level of nitrogen application, there was no discernible difference after N2 treatment. Correspondingly, the highest dry herbage and flos yield was also obtained from the same treatment (N2) (Fig. 1).

The nitrogen use efficiency (NUE) increased with all nitrogen applications (N0, N1, and N2) relative to N0 treatment (Fig. 2). However, there were no significant differences between N0 (20.8 mg g−1), N1 (22.1 mg g−1), and N2 (23.0 mg g−1) treatments. An increase in nitrogen use efficiency with increasing the nitrogen applications is reported for Achillea collina Becker ex Rchb. (15). The data of these authors show that the nitrogen uptake of the plant was 28.8 mg under nitrogen-deficient conditions and 36.8 mg under nitrogen-sufficient conditions to produce 1 g of dry matter. Our results clearly agree with this study. On the other hand, the different times of nitrogen application did not significantly affect the total nitrogen content and nitrogen use efficiency (Fig. 2).

Antioxidant activity and flavonoid content

The chemical and biological properties of medicinal plants mostly change depending on the growing conditions, environmental effects and genetical characteristics of the species (22). Although several studies have been performed about the environmental impact on the biochemical properties of some medicinal plants, there is limited information about the effect of nitrogen fertilization on the antioxidant activity of Achillea millefolium L. In the present study the highest antioxidant activity (451.9 µmol FRAP/g DM) was obtained in the N2 treatment, whereas the lowest values were recorded for N0 (413.1 µmol FRAP/g DM) and N3 (395.7 µmol FRAP/g DM). Nitrogen application at sowing (A1) and at the vegetative stage (A2) led to a significant increase in the antioxidant activity in comparison to that observed after application at the pre-bloom stage (A3) (Fig. 3). The flavonoid content of the plants varied between 76.6 mg rutin/g DM and 86.7 mg rutin/g DM. However, both factors, nitrogen amount and application time, did not have significant effects on the flavonoid content of the plants (data not shown). A previous study on Achillea collina
Becker ex Rchb. (15) showed that nitrogen starvation may increase the antioxidant activity compared to nitrogen-sufficient conditions. Studies on *Matricaria chamomilla* L. plants also reported a decrease in antioxidant activity with increasing the soil nitrogen content (19). Accordingly, the highest antioxidant activity in the present study was observed in nitrogen-deficient conditions. The lack of significant alterations in the flavonoid content suggests that the change of the antioxidant activity is a result of other enzymatic or non-enzymatic factors such as the phenylalanine ammonia lyase (PAL) and hydroxycinnamic acid derivatives, as shown by Giorgi et al. (15). The authors observed a significant accumulation of 3-O-caffeoyl quinic acid (chlorogenic acid), and 3,5- and 4,5-di-O-caffeoyl quinic acids in nitrogen-deficient *Achillea collina* Becker ex Rchb. plants accompanied by an increased activity of PAL, a key enzyme in the phenylpropanoid biosynthesis. Similar results were reported about nitrogen-deficient *Matricaria chamomilla* L. plants (19).

![Fig. 3](image-url)

**Fig. 3.** Effects of different nitrogen amounts (0 [N₀], 0.45 [N₁], 0.9 g [N₂], and 1.35 g [N₃] per pot) and application times (A₁: at sowing, A₂: at the vegetative stage, A₃: at the pre-bloom stage) on the content of essential oil components (peak area, % of total) in dry flocc of cv. Proa plants.

**Rate and composition of the essential oil**

The effect of nitrogen application on the rate of essential oil production has been investigated for different medicinal plants. Higher amounts of nitrogen lead to a decrease of the essential oil rate of *Origanum vulgare* L. (3), whereas no variation was detected in *Artemisia annua* L., *A. absinthium* L., and *Matricaria chamomilla* L. (20). Mansour and Ali (20) revealed that various nitrogen treatments may not affect the chamazulene content of *Artemisia absinthium* L. In the present work chamazulene was either negatively (N₁, N₂) or not influenced (N₃) by the nitrogen supply. However, a little increase could be noted with the application time of nitrogen closer to the blooming stage (Table 1). Variable tendencies were observed with regard to the other essential oil components, as affected by the nitrogen regime (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Components of the essential oil</th>
<th>Nitrogen amounts</th>
<th>Application times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N₀</td>
<td>N₁</td>
</tr>
<tr>
<td>Sabinene + β-Pinene</td>
<td>1.1</td>
<td>8.3</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>1.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Artemisia ketone</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>4.5</td>
<td>3.0</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>β-Caryophyllene</td>
<td>11.4</td>
<td>9.0</td>
</tr>
<tr>
<td>α-Humulene</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>3.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>6.9</td>
<td>5.2</td>
</tr>
<tr>
<td>Chamazulene</td>
<td>36.7</td>
<td>28.1</td>
</tr>
</tbody>
</table>

There is limited information on the effect of different nitrogen amounts on the essential oil composition of *Achillea millefolium*. Mansour and Ali (20) revealed that various nitrogen treatments may not affect the chamazulene content of *Artemisia absinthium* L. In the present work chamazulene was either negatively (N₁, N₂) or not influenced (N₃) by the nitrogen supply. However, a little increase could be noted with the application time of nitrogen closer to the blooming stage. Variable tendencies were observed with regard to the other essential oil components, as affected by the nitrogen regime (Table 1).

**Conclusions**

As medicinal plants are generally collected from the wild flora, it is of fundamental importance to identify the optimum growth conditions for increasing their yield and quality potential. Our research suggests a general trend of inverse relation between the aromatic type components – antioxidants (most probably hydroxycinnamic acids), essential oil, chamazulene, and the degree of nitrogen supply. This could be accounted for by the competition of aromatic and nitrogen metabolism for common carbon precursors. Nevertheless, the data point to the nitrogen regime being a subtle tool to regulate plant performance by improving dry matter production without loss of essential oil.
rate and with no significant damage to chamazulene content and antioxidant activity. Our results are generally in agreement with research on other medicinal plants; the diversity of data could be due to specificities of plant species, organ, tissue, developmental stage, as well as experimental conditions such as amount, time and duration of nitrogen application.

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REFERENCES