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INVESTIGATION OF ABIOGENIC STRESS-INDUCED ALTERATIONS IN THE LEVEL OF SECONDARY METABOLITES IN POPPY PLANTS (*PAPAVER SOMNIFERUM* L.)

BEÁTA SZABÓ,[1]* Á. LAKATOS,[2] T. KŐSZEGI[2] and L. BOTZ[3]

[1] Department of Botany, Faculty of Sciences, Pécs University, Ifjúság u. 6, H-7624 Pécs, Hungary
[2] Department of Clinical Chemistry, Medical School, Pécs University, Ifjúság u. 13, H-7624 Pécs, Hungary
[3] Pharmaceutical Institute, Medical School, Pécs University, Honvéd u. 3, H-7624 Pécs, Hungary

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We aimed to understand the effects of water stress on the alkaloid production in various developmental stages of poppy plants and the effect of stress on the alkaloids content in the capsules. Three stages of the life cycle of *Papaver somniferum* L. were selected in our studies: Rosette, Flowering and Lancing developmental stages. Four types of water conditions were examined: Control, Withdrawal of Water, 50% Water Supply and Inundation.

The morphological monitoring, results of Relative Water Content and proline content were used as indicators of stress. The result of the measurements in poppy leaves show that the secondary metabolites dramatically respond to these stress conditions. The constant water supply was beneficial for the accumulation of alkaloids in the capsules.

Keywords: Papaver somniferum L. – alkaloids accumulation – growth – water stress condition

INTRODUCTION

Many alkaloids are pharmacologically active substances that possess various physiological activities in humans and animals. The largest and most diverse group of alkaloids is the group of benzylisoquinolines with about 2500 members. Some important members are present in the opium poppy, *Papaver somniferum* L. (*Papaveraceae* family). Opium poppy, which contains more than 50 different alkaloids, remains one of the most important industrial and medicinal plants. The stress

*Corresponding author; e-mail: csele@pointer.hu

Abbreviations. DW: Dry weight; HPLC: High performance liquid chromatography; HPLC-DAD: High performance liquid chromatography – diode-array detection; Rpm: Rotation per minute; RWC: Relative Water Content; TLC: Thin layer chromatography; TW: Turgid weight; UV: Ultra violet; VIS: Visible; W: Fresh weight.

factors greatly influence the growth of plants and on the productivity of crops. The ability of plants to adapt to environmental stress conditions is genetically determined and includes biochemical, molecular biological and physiological components. The alkaloid content of *Papaver somniferum* L. has been examined previously by Bernáth and Tétényi [2]. They investigated the effect of different sowing-times and growing areas on dry matter and alkaloid production. The autumn sowing and the 20 × 20 cm plant spacing were favourable on alkaloid production. Moreover, they studied the effect of environment factors (light, temperature and nutrition) on the growth and the alkaloid production of poppy. According to their observations the maximum of dry matter was produced in a low temperature environment, but higher light intensity (3.2×10^4 lux) and the total amount of alkaloids reached a maximum value in a high temperature regime under the 3.2×10^4 lux illumination [1]. Ilinskaya and Yosifova [3] investigated the influence of climatic conditions on the alkaloid contents. They examined the three varieties of opium poppy in different regions. The main climatic factors are temperature and humidity. The morphine content decreases from South to North direction. Turkhede and Rajat De-Singh examined soil-moisture stress and water-utilization efficiency on the opium yield of the poppy at different stages of growth. The water deficiency did not result in significant changes in the morphine content [11]. Kaicker investigated the environmental effects on morphine content in *Papaver somniferum* L. over a five-year period. The environmental factors -temperature and humidity – may affect enzymatic pathways of morphine biosynthesis [4].

We examined the effect of drought and water stress conditions on the contents of secondary metabolites during various developmental stages of poppy plants and the stress effects on the alkaloid content in the capsules. In general, the alkaloid contents of plants are very low therefore we were looking for simple, fast and specific analytical methods for quantitative and qualitative analyses of alkaloids of leaf extracts of poppy plants grown under stress conditions. The HPLC technique with REMEDI HS™ offers sensitive detection and identification of chemicals in biological specimens [9].

The aims of our study were to investigate the effects of drought and water stress on the alkaloid production during various developmental stages of poppy leaves (rosette and flowering stages). We investigated the effects of abiogenic stress on the alkaloid production in capsules.

We also measured stress indicators like morphology, concentration of proline and Relative Water Content (RWC).

MATERIALS AND METHODS

Plant material and growth condition

Papaver somniferum L. (Buddha-BS/04/2F) plants were grown in laboratory conditions (phytotron: Conviron, CMP 3244, Canada, temperature: 20 °C, light: 30,000 lux; Research Institute for Medicinal Plants, Budakalász, Hungary). The potting mix-

ture we used was: 50% turf, 25% organic fertilizer and 25% sand. The composition of soil: 20 mg/100 g N, 50–80 mg/100 g P_2O_5, 200–300 mg/100 g K_2O, and we use chemical fertilizer (Wuxal Super, Agro-Pack[96] Ltd.) and boron-solution (0.50 ml/50 ml/pot, FitoHorm, Baja). The experiment comprised 22 treatments (M1-M22) to compartmentalize (Table 1). The irrigation schedule was implemented at three stages (Rosette: 60–69 days, Flowering: 105–112 days and early Lancing: 130–138 days) of the life cycle of *Papaver somniferum* L. Four types of water condition groups were examined: Control (M1), withdraw of water (M2–M8), 50% water supply (M9–M15) and inundation (M16–M22). For example in case of M2 the plants did not receive water supply in rosette stage and received optimum water supply in flowering and lancing stages (– + +); in case of M12 plants received 50% water supply in rosette and flowering stages and optimum in lancing stage (50% 50% +). The control plants received water according to the their needs. During drought stress the water supply was withdrawn. In case of 50% irrigation the plants received half of the amount of

Table 1
The model of three types stress conditions
in three development stages of *Papaver somniferum* L.

Treatments	Rosette stage (60–69 days)	Flowering stage (105–112 days)	Lancing stage (130–138 days)
M1	+	+	+
M2	–	+	+
M3	+	–	+
M4	+	+	–
M5	–	–	+
M6	–	+	–
M7	+	–	–
M8	–	–	–
M9	50%	+	+
M10	+	50%	+
M11	+	+	50%
M12	50%	50%	+
M13	50%	+	50%
M14	+	50%	50%
M15	50%	50%	50%
M16	I	+	+
M17	+	I	+
M18	+	+	I
M19	I	I	+
M20	I	+	I
M21	+	I	I
M22	I	I	I

+: control (M1)
–: water withdrawal (M2–M8)
50%: half of control water-supply (M9–M15)
I: inundation (M16–M22)

control water supply. The plants were irrigated by optimum needs among the treatments. The experiment was applied in 50 pots. The samples were taken after the treatments. In the rosette and flowering stages the leaves of poppy plants were harvested from three or four plots and the alkaloid content was analyzed. The measurements of alkaloid contents of poppy were carried out from capsules after treatments in the Lancing developmental stage. We performed the experiment in two series.

Measurement of Relative Water Content (RWC)

We prepared 1.6 cm diameter discs from fresh leaves. After the samples were hydrated to full turgidity for 24 hours under normal room light and temperature, we weighed their fresh weight (W). After 24 hours the samples were taken out of water and were blotted dry of any surface moisture quickly with filter paper and immediately weighed (TW). Samples were then oven dried at 80 °C for 72 hours and weighed dry weight (DW). We carried out two parallel measurements. The Relative Water Content (RWC) was calculated according to this equation:

$$\mathrm{RWC}(\%) = [(\mathrm{W} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW})] \times 100$$

Measurement of proline content

Leaf samples (0.20 g) were powered with liquid nitrogen and were extracted with 4.0 ml 3-sulphosalicyclic acid, then the suspension were centrifugated for 10 minutes (3000 rpm). The supernatant of extracts was filtered. 2.0 ml of this filtered extract was reacted with 2.0 ml glacial acetic acid and 2.0 ml of acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid plus 20 ml 6 M phosphoric acid; this mixture is stable at 4 °C, for 24 hours) for 1 hour at 100 °C in a boiling test tube. The mixture is then cooled in an ice bath and 4.0 ml of toluene added. After vigorous mixing the two layers separated out and the pink-red colour at the top could be removed with pipette. We measured the proline content at 520 nm with spectrophotometer (Hitachi U-2000, Kentucky, USA). We carried out two parallel measurements.

Leaf samples for HPLC analysis

Samples of 1 g of *Papaver somniferum* L. leaves were placed in 30.0 ml methanol then were turbo-extracted (POLYTRON Turbo Extractor). The extract was filtered through filter paper and was acidified by addition of 5.0 ml distilled water and two drops of concentrated HCl. This solution was further concentrated to an end volume of 5.0 ml in a distillator equipment (BÜCHI). After filtration the extract was diluted to 5.0 ml with distilled water. The leaf samples were applied to the broad-spectrum

HPLC analyser with coupled-column system (REMEDi HS™ Drug Profiling System, BIO-RAD). To extract, purify and analyze drugs, we used liquid chromatography with on-line sample analysis and multicolumn technique. The instrument used multi-wavelength (between 200 nm to 300 nm) UV detection. The sample spectra were then automatically identified by using the library of known drug spectra stored in the memory of the computer [8]. 1.0 ml of the extract was mixed with 200.0 ml of internal standard solution (Bio-Rad standard). After brief centrifugation the sample was placed in the HPLC analyzer. After automated injection the sample is combined with a buffer and passed through four cartridges. The purification cartridge concentrates samples and separated them from interfering proteins and salts. When the drugs were eluted from this cartridge a mobile phase was introduced. The mobile phase was aqueous phosphate buffer solution with less than 40% acetonitrile that is a ready-to-use eluent manufactured by BIO-RAD Laboratories, Hercules, CA, US (Catalog: 195-7114). The mobile phase drove the drugs through the extraction cartridge. The endogenous organic acids were retained in this cartridge while the weak acidic, neutral and alkaline drugs passed through. The third cartridge, the Separation I Cartridge, was a reverse phase cartridge that separated the weak alkaline compounds. The fourth cartridge, Separation II Cartridge, differentiated alkaline compounds by cation exchange. All separations were isocratic. A conditioning cartridge is applied to saturate the mobile phase with silica. This prevents the two separation cartridges to be dissolved [10]. We carried out 4 parallel measurements for all types of treatments. Standards were: *Morphinium chloratum* (Ph.Hg.VII.),

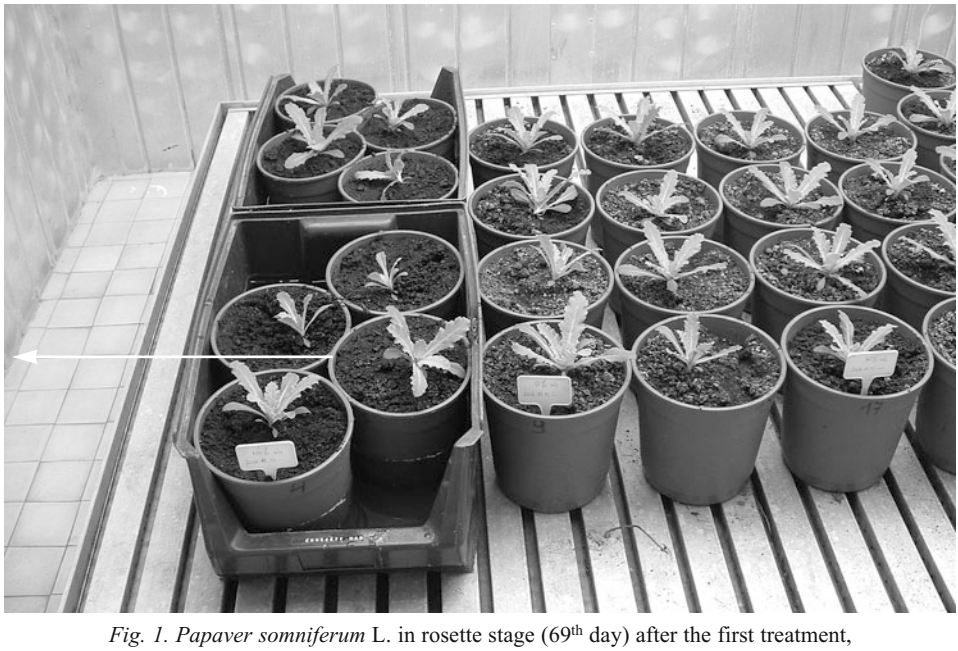

Fig. 1. Papaver somniferum L. in rosette stage (69th day) after the first treatment,
A: after inundation leaves were withered

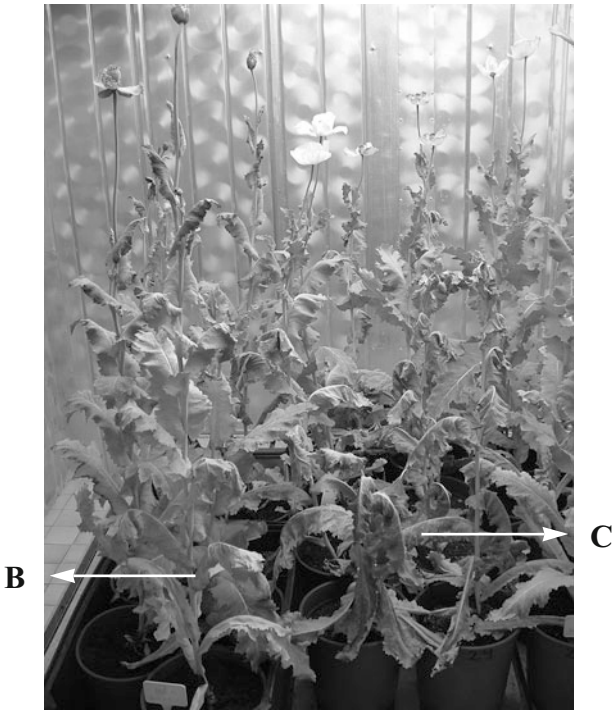

Fig. 2. Papaver somniferum L. in the end of flowering stage (112th day) after the second treatment, B: after inundation and C: after water withdrawal the leaves were withered

Codeinum chloratum (Ph.Hg.VII.), Thebaine (Research Institute for Medicinal Plants, Budakalász), *Papaverinum chloratum* (Ph.Eur.IV.), *Narceinum hydrochloratum* (Ph.Hg.V.), *Narcotinum hydrochloricum* (Ph.Hg.V.).

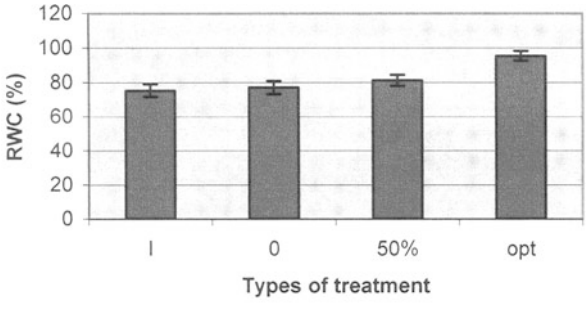

Fig. 3. Results of RWC after first treatment (in rosette stage). I: inundation, 50%: 50% water-supply, 0: water withdrawal, opt: control

Preparation of capsule samples for HPLC analysis

Plant samples (200 ± 2 mg) were powdered and placed in 50.0 ml 0.1M HCl solution then were shaken for 2 hours. After this the extracts were filtered. Then analysis was carried out with Agilent 1100 HPLC-DAD apparatus (Eurosper-100C18 column 120 mm × 4 mm; 5 μm), at flow rate 1 ml/min and temperature 40 °C. We applied gradient elution with the following components: A: water + trifluoroacetate (993.3 + 6.7), gradient component B: methanol + acetonitrile + water + trifluoroacetate (770.0 + 24.6 + 198.7 + 6.7) (Merck, Reanal). The gradient operation program was the following: gradient component B: 16.0%–54.8%/14.4 min, 54.8%–100.1%/0.1 min, 100.0%/1.9 min, and 100.0%–16.0%/0.1 min. Before injection the column was conditioned with gradient component B for 4.0 min. The detection wavelength was λ = 288 nm. We carried out 2 parallel measurements for all types of treatments.

RESULTS

In our experiments we measured the effects of the stress conditions (morphological parameters and RWC and the concentration of proline).

Morphological observations

The morphology of the plants under stress condition was greatly affected. In the case of inundation the leaves were withered in rosette stage (Fig. 1). In the flowering stage the leaves were withered during inundation and water withdrawal (Fig. 2).

Leaf of Relative Water Content

The results of RWC indicated the onset of stress condition. After the first treatment (rosette stage) with 20–25% lower value of RWC was measured when plants were grown under non-optimal growing condition (inundation, 50% water-supply and water withdrawal, Fig. 3). We detected 2.5–3.5 times lower value RWC after the second treatment (flowering stage) during inundation and water withdrawal (Fig. 4).

Leaf of proline content

The results of proline contents also responded to the stress condition. We measured 6.6 times higher values than in the control plants after inundation at rosette stage (inundation: 268.10 μg/g, control: 40.75 μg/g fresh matter). The values of other types of treatment (50% water-supply and water withdrawal) were similar to the control groups. After flowering (second treatment) we measured 5.0 times higher proline

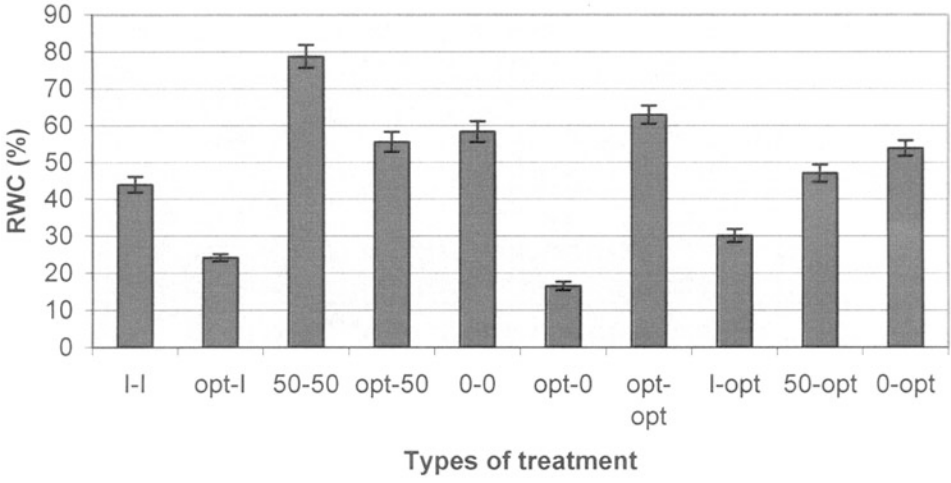

Fig. 4. Results of RWC after second treatment (in flowering stage). The types of combination of two treatments (I-I, opt-I, etc. – I: inundation, 50%: 50% water-supply, 0: water withdrawal, opt: control)

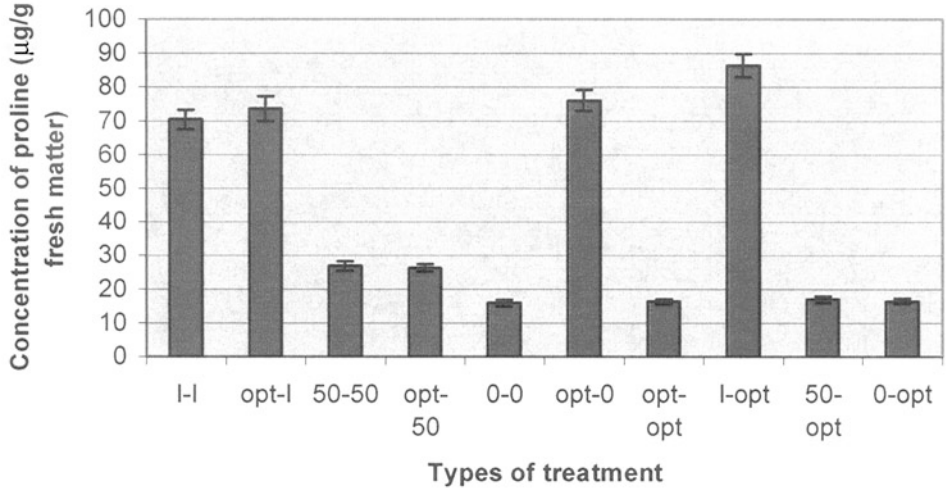

Fig. 5. Results of measurement of proline concentration after second treatment (in flowering treatment). The types of combination of two treatments (I-I, opt-I, etc. – I: inundation, 50%: 50% water-supply, 0: water withdrawal, opt: control)

content by inundation and water withdrawal in the leaves compared with the control experiment (Fig. 5).

Analysis of alkaloid contents of poppy leaves

Figure 6 shows the ratio of morphine, thebaine and codeine in leaves at rosette stage whereas Table 2 presents the absolute values of alkaloid contents in poppy leaves. We measured higher proportion of morphine at this stage (the Buddha-BS/04/2F species has high morphine content-2.4%). The quantity of the three alkaloids (morphine, thebaine and codeine) were lower than in the control leaves in almost all treatments.

Table 2
Results of alkaloid content after first treatment (in rosette stage)

	I (µg/g fresh matter) ±SD	0 (µg/g fresh matter) ±SD	50% (µg/g fresh matter) ±SD	opt (µg/g fresh matter) ±SD
Morphine	638.78±31.94	703.01±28.12	562.40±33.74	1207.06±60.35
Thebaine	11.14±0.56	1.65±0.12	6.95±0.42	36.93±1.85
Codeine	26.16±1.05	15.18±1.06	10.29±0.82	26.13±1.16

I: inundation, 50%: 50% water-supply, 0: water withdrawal, opt: control

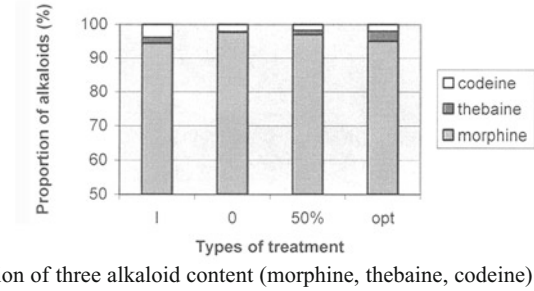

Fig. 6. Proportion of three alkaloid content (morphine, thebaine, codeine) in rosette stage

During inundation and water withdrawal at the flowering stage (after second treatment) we found 5–8 times higher alkaloid contents than in the control leaves (Fig. 7). All three alkaloids responded to stress condition in a similar fashion. The data of the alkaloid contents corresponded to the results of RWC, proline and morphological changes.

Analysis of alkaloid contents of capsules

The other aim of our study was to investigate the effects of drought and water stress on the alkaloid production in capsules by different irrigation in three development stages (rosette, flowering and lancing). These development stages were identified according to earlier observations [11].

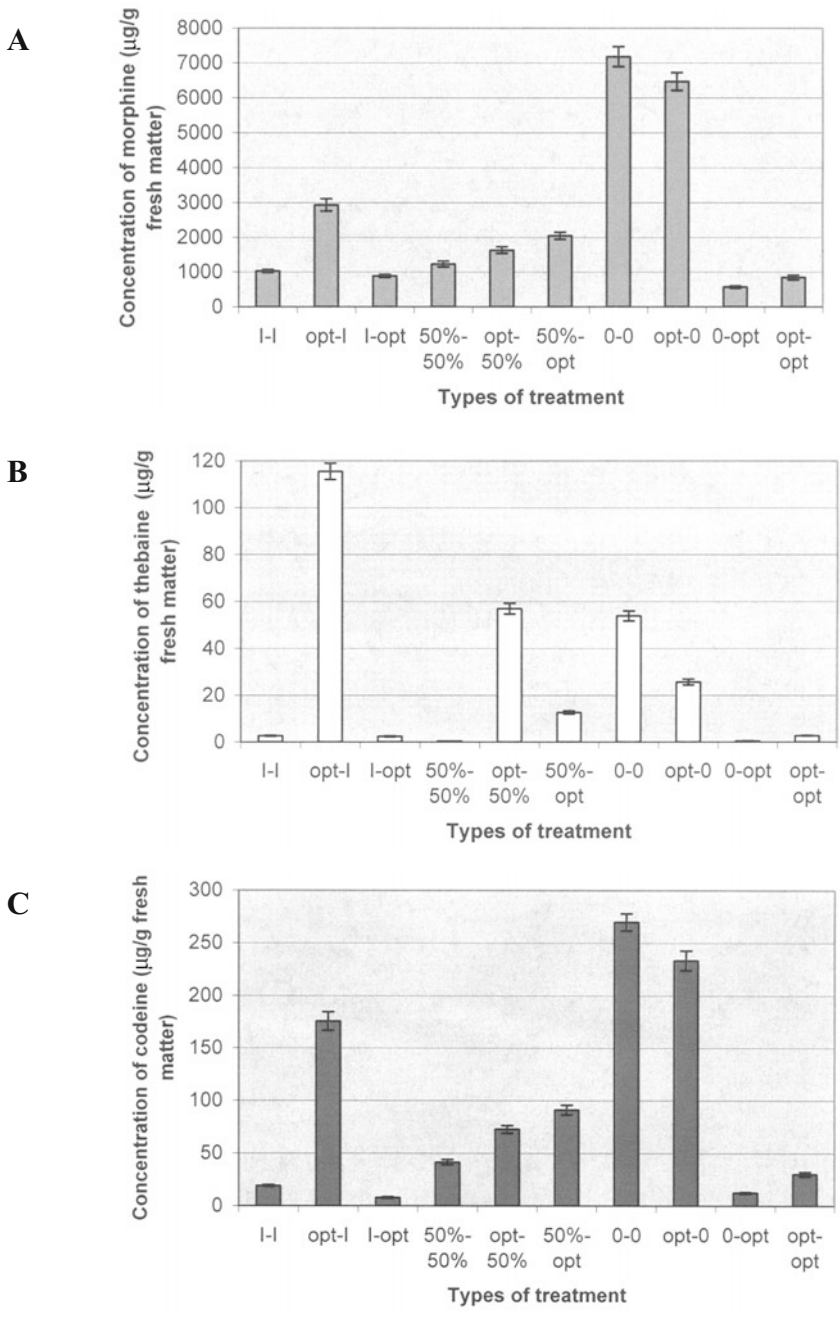

Fig. 7. Concentration of alkaloids (A: morphine; B: thebaine; C: codeine) after second treatment (flowering stage). The types of combination of two treatments (I-I, opt-I, etc. – I: inundation, 50%: 50% water-supply, 0: water withdrawal, opt: control)

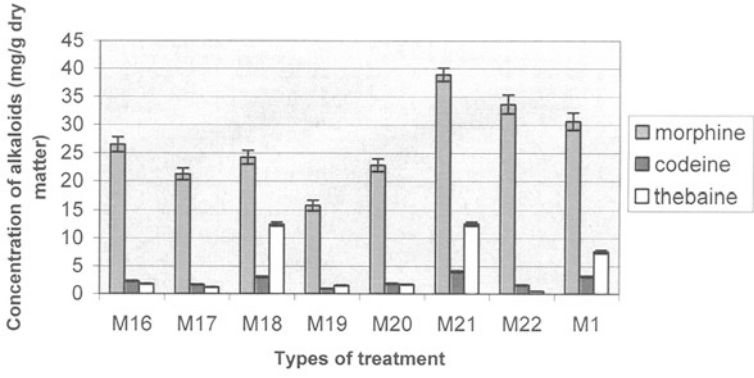

Fig. 8. Alkaloid content of capsules at inundation

Figures 8, 9 and 10 illustrate the three types of treatments. The amount of thebaine (0.51–13.85 mg/g) and morphine (9.25–38.93 mg/g dry matter) change dramatically by various irrigation regimes. In general the quantity of the three alkaloids (morphine, thebaine and codeine) showed 20% lower values than the control in almost all treatments. When the water conditions were altered only at a single developmental stage, we detected 10–20% higher values of alkaloids for all treatments. The frequency of water supply had the greatest impact on the alkaloid content. We observed extremely high morphine values during water withdrawal in treatment M3 (34.29 mg/g), during 50% irrigation in treatment M11 (35.11 mg/g) and during inundation in treatment M21 (38.93 mg/g) (Fig. 10).

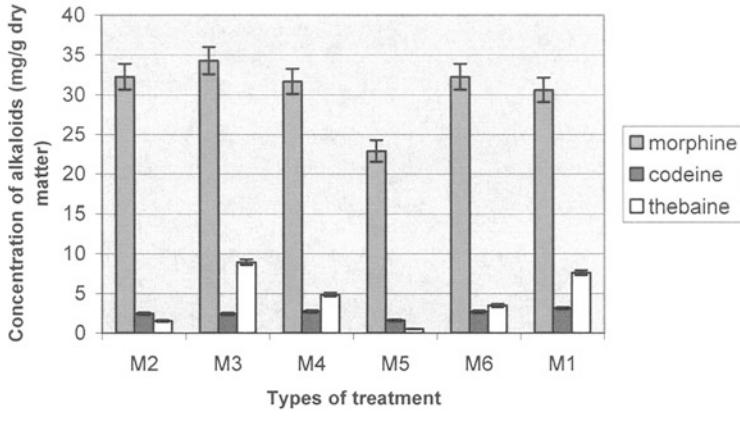

Fig. 9. Alkaloid content of capsules at water withdrawal

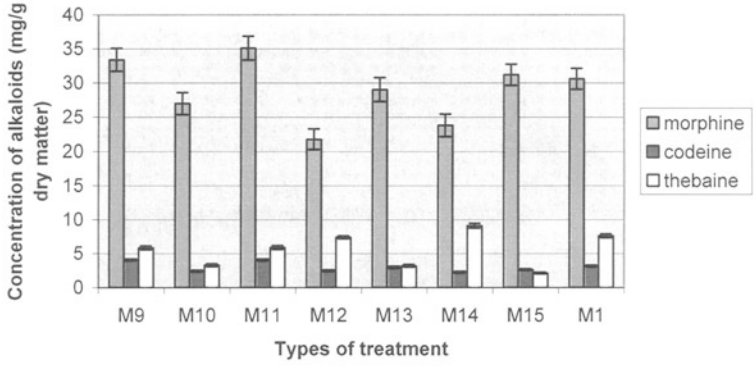

Fig. 10. Alkaloid content of capsules at 50% water-supply

DISCUSSION

Many species of higher plants are major sources of natural products used in the pharmaceutical, agrochemical and food industries. On one hand, the breeding work of poppy has been focused on increasing the production and alkaloid levels of the capsules and producing a desired spectrum of alkaloids for pharmaceutical processing. On the other hand, food producers aim is to develop cultivars with low alkaloid content. Extreme environmental conditions affect plant growth and development and through altering gene expression and cellular metabolism. The stress factors (light, temperature, nutrition) are associated with dry matter production, the size of the capsule and its alkaloid content. The highest morphine content was observed in the capsules at medium nitrogen (N) level and limited supplementary potassium oxide (K_2O) and phosphoric acid (P_2O_5) [7]. Adequate rainfall before flowering, followed by dry, warm weather, increased the morphine content of capsules [4]. Penka examined the effect of water supply at germination, rosette formation, stem branching and flower-bud formation developmental stages of poppy plants. When irrigation was used during the developmental stages of flowering and ripening as well, growth of the capsules being formed was stimulated, and the relative content of morphine therein likewise. During the later stages of ripening, lower contents of morphine were found in the capsules of irrigated plants, in comparison with non-irrigated plants [5]. The results of earlier research in water supply are contradictory. In earlier studies the water-utilization efficiency on the opium yield of the poppy at different stages of growth (rosette, lancing) was investigated by Turkhede and Singh [11]. They performed their experiments on cultivated land from where they collected capsules and they measured the quantity of opium and morphine.

Our objective was to understand the effects of water stress on the alkaloid production in various developmental stages of poppy plant and the stress effect on the alkaloids content in the capsules. The water deficiency did not result in significant changers in the morphine content.

We analyzed the alkaloid content from poppy leaves in rosette and flowering stages. The plant material we used came from natural habitats. Therefore we had to overcome the problem of the high number of coloured spots after a TLC separation [8]. We wanted to select a suitable detection method for our examination. We calibrated the standard solutions with three methods (VIS, UV, fluorescence). The fluorescence method filtered out coloured substances in our analysis, however this technique does not eliminate all interfering substances. The HPLC technique (REMEDi HS^{TM} Drug Profiling System) is a simple, fast and specific analytical method for quantitative and qualitative analysis of alkaloids of leaf extracts of poppy plants grown under stress conditions. We could detect morphine, thebaine and codeine alkaloids in extracts of leaves. This method is suitable to compare various stress effects [10].

We examined the morphine alkaloids in the leaves and the results indicate that the stress conditions affected the synthesis of alkaloid content (morphine, codeine, thebaine) in the growth. All three alkaloids change unison under stress condition in the samples of leaves. The inundation and water withdrawal also stimulated the alkaloid content in the leaves (Fig. 7). We received these results after the second sampling in the flowering stage. We received salient value in case of optimum water supply in rosette stage but inundation in flowering stage (opt-I), water withdrawal both rosette and flowering stage (0-0) and optimum irrigation in rosette and water withdrawal in flowering stage (opt-0).

The proline has function in the protection of water stress. The results of proline content prove the onset of stress conditions. Similarly the determination of RWC demonstrates it. We found that results of these molecules and alkaloid content of poppy leaves are corresponding. The stress condition has significant influence on the secondary metabolites in the poppy.

The change of quantity of morphine alkaloids in the capsules varied from the results of leaves. The alkaloid content increased at constant water supply in the capsules (M3, M11, M21), however the variation of irrigations in all the three development stages decreased it, for example M17, M14, M12, M5 (Figs 8–10).

CONCLUSIONS

In our experiments, the morphological observations and the results of relative water and proline contents indicate the onset of stress condition. The water stress condition affect the accumulation of alkaloids in leaves during plant growth. As well as, the constant water supply was stimulated the accumulation of alkaloids in the capsules.

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REFERENCES

1. Bernáth, J., Tétényi, P. (1981) The effect of environmental factors on growth. Development and alkaloid production of poppy (*Papaver somniferum* L.) II. Interaction of light and temperature. *Biochem. Physiol. Pflanzen 176,* 599–605.
2. Bernáth, J., Tétényi, P. (1982) Production characteristics of *Papaver somniferum* L. Cultivars of different origin and vegetation cycles. UNODC-Bull. *Narcotics 34,* 113–127.
3. Ilinskaya, T. N., Yosifova, M. G. (1956) Influence of the conditions under which the poppy is grown on the alkaloid content of the opium obtained. *Bull. Narcotics 3,* 38–41.
4. Kaicker, U. S., Saini, H. C., Singh, H. P., Choudhury, B. (1978) Environmental effects on morphine content inopium poppy (*Papaver somniferum* L.). *Bull. Narcotics 3,* 69–74.
5. Penka, M. (1968) Changes in water economy and morphine content in irrigated and non-irrigated plants of *Papaver somniferum* L. *Acta Univ. Agricult. 4,* 579–590.
6. Sárkány, S., Bernáth, J., Tétényi, P. (2001) The poppy. Flora of Hungary, V. Press, 22, Akadémiai Kiadó, Budapest. (In Hungarian)
7. Schroder, H. (1966) Der Einfluss von Mineraldüngung und Standort auf Morphingehalte sowie andere qualitative und quantitative Merkmale des Mohns (*Papaver somniferum* L.). *Pharmazie 21,* 635–641.
8. Szabó, B., Lakatos, Á., Kőszegi, T., Botz, L. (2003) Thin-layer chromatography-densitometry and liquid chromatography analysis of alkaloids in leaves of *Papaver somniferum* under stress conditions. *J. Planar Chromatogr. 16,* 293–297.
9. Szabó, B., Tyihák, E., Szabó, L. Gy., Botz, L. (2003) Mycotoxin and drought stress induced change of alkaloid content of *Papaver somniferum* plantlets. *Acta Bot. Hungarica 45,* 409–417.
10. Szabó, B., Lakatos, Á., Kőszegi, T., Kátay, Gy., Botz, L. (2005) TLC-densitometry and HPLC analysis of alkaloids in leaves of *Papaver somniferum* under stress conditions. *J. AOAC Int. 88,* 1571–1577.
11. Turkhede, B. B., Rajat, De Singh, R. K. (1981) Consumptive water use by opium poppy. *Indian J. Agric. Sci. 51,* (2), 102–107.