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ACCUMULATION OF TRIGONELLINE IN THE LEAVES OF HEMP (*CANNABIS SATIVA*) IN RESPONSE TO WATER-DEFICIT STRESS

Short communication

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ABSTRACT

Trigonelline (TRG) has been proposed as a molecular marker of water-deficit stress in plants. This study evaluated whether trigonelline accumulates in hemp (*Cannabis sativa* L.) leaves under water-deficit conditions and whether responses differ between cultivars. Two cultivars ('Jin Ma' and 'NWG 2730') were introduced to water-deficit stress in a controlled greenhouse experiment by withholding irrigation. Trigonelline was detected in leaves of both cultivars and increased by 451% under water-deficit stress, rising from 0.25 ± 0.08 μmol·g^{-1} FW to 1.14 ± 0.56 μmol·g^{-1} FW. Leaf relative water content (RWC) decreased by 58.8%. No significant differences were observed between cultivars for trigonelline concentration or relative water content. These results demonstrate that trigonelline accumulation is a robust physiological response to water-deficit stress in hemp, independent of cultivar. Trigonelline is a reliable and practical biochemical indicator for early drought stress monitoring in commercial hemp production systems.

Key words: leaf relative water content, trigonelline content

INTRODUCTION

Trigonelline (TRG), N-methylnicotinic acid, was first detected in *Trigonella foenum-graecum* L. seeds (Jahns 1885) and has since been found in seeds, leaves, roots, stems, and flowers of a wide range of plants in a range of 0.3–13.0 μmol·g^{-1} FW (Chen & Wood 2004; Ashihara & Watanabe 2014; Schwartz et al. 2014). The pyridine-nucleotide cycle produces nicotinic acid, which is synthesized by TRG (Zheng et al. 2004). TRG acts as a drought-stress marker and a regulator of the cell cycle while responding as a solute to drought stress (Chen & Wood 2004). As a storage form of nicotinic acid, TRG can be demethylated, thereby reentering the nicotinamide metabolic pathway (Cho et al. 2002). The effect of several abiotic stresses on TRG content, including salinity, temperature, and drought, on TRG accumulation and synthesis (Boyer 1982) has been characterized. Moreover, although TRG has been identified in the inflorescence of hemp (*Cannabis sativa* L.) (Spano et al. 2021), it has not been reported to occur in hemp leaves, even though this plant produces a variety of secondary metabolites. In total, more than 550 chemical compounds in hemp have been identified, of which 125 are cannabinoid compounds (Fordjour et al. 2023; Wishart et al. 2024). Commercial hemp cultivation (with a tetrahydrocannabinol concentration of less than 0.3% by dry weight) is expanding into environments where short- and long-term water limitation is common (Morgan et al. 2024), substantially affecting production.

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A previous study on hemp demonstrated that severe water deficit or drought significantly affects yield and cannabinoid concentration. This underscores underscoring the need to improve the physiological management and to discover new cultivars with water-stress resilience (Morgan et al. 2024). Even though studies have investigated the response of hemp to water-deficit stress at the transcriptomic level (van Bakel et al. 2011), it remains difficult to identify robust biochemical indicators that can quantify water-deficit intensity independent of cultivar differences. Hence, identifying stress-responsive metabolites that change early and significantly under water-deficit conditions would be a useful tool for improving crop management and breeding protocols (Gao et al. 2018).

Variation in the accumulation of TRG in response to soil moisture has been reported in five different cultivars of the grass *Panicum virgatum* L. Schwartz et al. (2014) showed that under drought stress conditions, the upland cultivars ('Blackwell', 'Cave in Rock', and 'Trailblazer') of *P. virgatum* contained a higher amount of TRG compared to the lowland cultivars ('Alamo' and 'Kanlow'). However, high moisture levels led to higher detectable TRG levels in lowland cultivars than in upland cultivars, suggesting that the two cultivars differ in water-deficit tolerance or water efficiency. Differences in TRG in response to water deficit among cultivars of hemp are yet to be studied.

Throughout the life cycle of hemp, it uses well-developed control mechanisms, such as stress-responsive regulatory pathways, to activate defense mechanisms that regulate secondary metabolic pathways (Flores-Sanchez & Verpoorte 2008). Investigating into the TRG detection in hemp may provide a way to regulate and manipulate its biosynthetic pathways with probable therapeutic significance, for instance, anti-inflammatory, neuroprotective, or metabolic health interventions (Nguyen et al. 2024).

While Duong et al. (2023) found that water-deficit stress is unlikely to increase the THC level, it remains unclear whether the TRG level is influenced by water-deficit stress. Moreover, their study covers only two hemp cultivars, and hemp requires substantial water during growth, so it is important to study additional cultivars and focus on other hemp secondary metabolites, such as TRG. Hence, the outcome of such studies may link genetic information with hemp water-deficit stress, which will ultimately help in the production of commercial hemp cultivars.

Under both control and water-deficit stress conditions, leaf samples extracted from two different cultivars of hemp ('Jin Ma' and 'NWG 2730') were evaluated for TRG accumulation. This experiment tested the null hypothesis (H_0) that there is no significant differences in TRG accumulation in response to water deficit in the leaves among hemp cultivars in a greenhouse setting.

MATERIALS AND METHODS

The greenhouse experiment was conducted at the Tree Improvement Center (TIC) at Southern Illinois University Carbondale (SIUC) from November 30, 2023, to January 22, 2024. The study was involved inducing water-deficit stress by withholding water from two hemp cultivars ('JinMa' and 'NWG 2730'). 'JinMa' originates from China and is a fiber cultivar, whereas 'NWG 2730', bred in the USA, is grown for seed oil. Both cultivars are reported to have less than 0.3% THC, meeting the definition of hemp (Griffin et al. 2021).

After seedling emergence and establishment, the plants were transplanted into cylindrical containers (Stuewe and Sons, USA) with a diameter of 10.16 cm and a depth of 35.56 cm, filled with a 1:1:1 mixture of silt loam field soil, sand, and potting substrate. Each container (hereafter referred to as a pot) contained eight plants (0.008 m² surface area) and served as the experimental unit. Pots were arranged in a randomized complete block design, with blocks defined by replicate. The experiment consisted of two hemp cultivars × two water treatments × ten replicate pots per treatment combination, yielding a total of 40 pots. Minimum and maximum temperatures in the greenhouse were recorded daily. Light intensity was recorded at canopy height in the afternoon every 2–3 days using a LI-COR Light Meter (model LI-250) to quantify the maximum amount of light the plants received. The average daily high temperature was 29.51 °C, and the daily low temperature was 19.54 °C. Throughout the growing period, the average light intensity was 370.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants were watered with a hose water-breaker nozzle (Dramm Corporation, USA) twice a day, once at 8 am and again at 3 pm, until the soil mixtures in the pot were moist. Once plants were well established, moisture treatment began 45 days after transplanting,

i.e., January 15, 2024. Pots were watered as usual for the control treatment and were not watered for 7 days for the water-deficit stress treatment, consistent with the protocol used in Kasim et al. (2021). The treatment design was a fully factorial combination of hemp cultivars ('JinMa' and 'NWG 2730') with control and treatment (no water).

Relative water content (RWC) and TRG were measured seven days after the water-deficit stress treatment was imposed. RWC was used to measure the efficacy of the water-deficit treatment. To measure RWC, forty (40) fully expanded leaves were sampled. One fully expanded leaf per plant was sampled from the base of a randomly chosen plant stem in each pot. The leaves were placed into a tared flask containing chloroform and distilled water (3 : 2 v/v). The relative water content in each sample was calculated as RWC = (fresh mass − dry mass)/(water saturated mass − dry mass) ×100 (Schwartz et al. 2014). Tissue from the second fully emerged leaf from the bottom of the stem of each plant was extracted in MeOH at 48 °C in the dark, and quaternary ammonium compounds were isolated as described (Cho et al. 1999; Yang et al. 2020). TRG was purified by Dowex-1-OH^- and Dowex-50-H^+ ion-exchange chromatography. The purified fractions were air-dried and resuspended in 1 mL of purified H_2O. TRG was measured spectrophotometrically – UV-Vis spectrum (H_2O) nm: λ_{max}.e. 264 (2700), as described by Yuyama and Suzuki (1985), and quantified using authentic TRG standards (Sigma, USA). The calibration curves demonstrated linearity over the concentration range of 1–30 $\mu g \cdot mL^{-1}$ ($R^2 = 0.9835$).

Data analysis

A two-way ANOVA in R, version 4.2.2 (R Core Team 2022), was performed to test for significant differences in RWC and TRG between cultivars (2 treatment levels) and water-deficit stress (2 levels). The package 'car' (Fox 2007) was used to perform Type II sums of squares, which were used for the ANOVA analysis. The 'cld' function from the package 'multicompView' (Mangiafico 2016) was used to create a compact letter display that visually represented significant differences. The packages 'ggplot' (Wickham 2010) and 'ggthemes' (Arnold 2015) were used to generate a bar plot that visualized the mean RWC and TRG with a standard error bar with a significance letter display on top of the bar. The 'emmeans' package (Lenth 2016) was used to determine the least-squares means. To create a density plot of the TRG, the 'ggdensity' function in the 'ggpubr' package was used (Kassambara 2020). A value of 10 was added to the raw data before analysis, followed by using the 'moments' package (Asquith 2014) to measure skewness, prompting a log transformation (due to zero values) of the TRG data.

RESULTS

Trigonelline vs. Treatment

The water-deficit stress treatment led to a 451% increase in the TRG content of hemp leaves ($p = 0.01$) (Table 1). The water-deficit stress treatment had a mean TRG amount in the leaves of 1.14 ± 0.56 $\mu mol \cdot g^{-1}$ FW. Conversely, the control treatment had a mean TRG amount in the leaves of 0.25 ± 0.08 $\mu mol \cdot g^{-1}$ FW (Fig. 1A, 2). TRG amount was recorded, converted, and is reported as µmol per g of FW to allow comparison with other studies. However, neither hemp cultivar identity alone nor interaction with the water-deficit stress treatment had a significant effect on TRG.

Relative water content vs. Treatment

The water-deficit stress treatment led to a 58.38% reduction in the RWC of hemp leaves ($p = 0.01$) (Table 2). The water-deficit stress treatment had an RWC with a mean of 36.8 ± 14.8%. Conversely, the control treatment had an RWC with a mean of 63.0 ± 9.3% (Fig. 1B, 2). However, neither hemp cultivar identity alone nor interaction with the water-deficit stress treatment had a significant effect on RWC.

Table 1. ANOVA testing trigonelline of hemp in response to cultivar identity and water-deficit stress treatment

Effect	Sum Sq	Df	F value	p-value
Water-deficit stress treatment	0.92	1	6.64	0.01
Cultivar	0.02	1	0.17	0.68
Water-deficit stress treatment: cultivar	0.06	1	0.40	0.52
Residuals	4.68	34		

Table 2. ANOVA testing relative water content of hemp in response to cultivar identity and water-deficit stress treatment

Effect	Sum Sq	Df	F value	p-value
Water-deficit stress treatment	17189	1	6.78	0.01
Cultivar	409	1	0.16	0.69
Water-deficit stress treatment: cultivar	472	1	0.18	0.66
Residuals	91195	36		

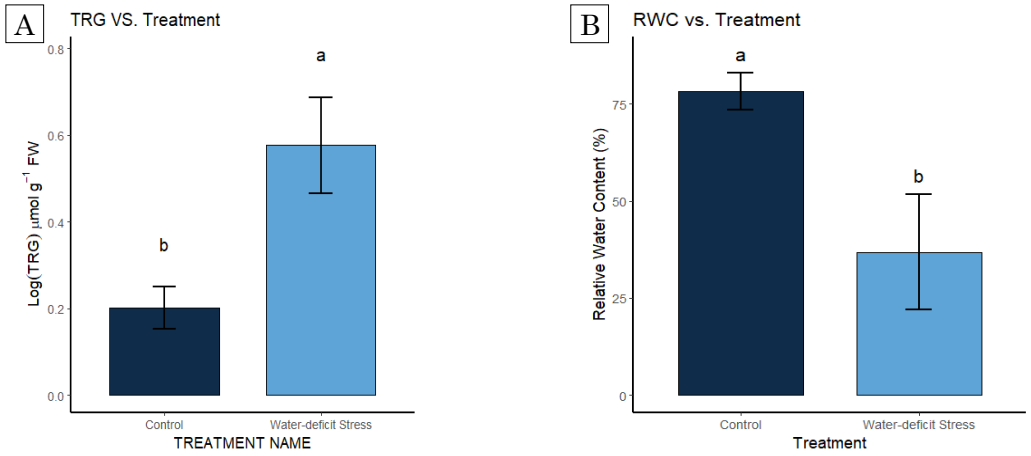


Figure 1. Log-transformed values of trigonelline (TRG) content (A) and relative water content (RWC) (B) in hemp leaves in the control and under water-deficit stress conditions

Means ± standard error (across cultivars, p = 0.01); Different letters (a and b) indicate significant differences between treatments; Error bars show the variability around the means

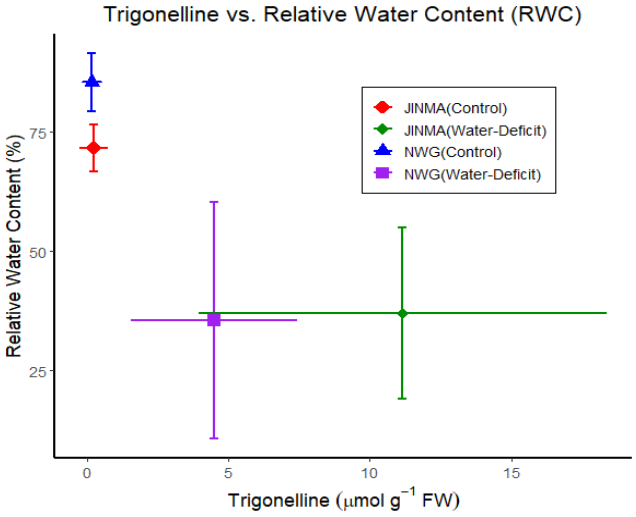


Figure 2. Trigonelline content and relative water content for each hemp cultivar in the control and under water-deficit stress conditions

Means ± standard error; Error bars show the variability

DISCUSSION

Hemp seeds, roots, and stems can accumulate TRG (Ashihara & Watanabe 2014). Still, this investigation is the first to test for TRG accumulation as a stress marker in hemp leaves under water deficit. Detectable amounts of TRG were observed in both fiber ('Jin Ma') and oilseed ('NWG 2730') cultivars of hemp. TRG levels were affected solely by water-deficit stress induced by withholding water. This finding demonstrates that water availability to hemp plants, rather than differences between the cultivars, affected TRG accumulation. The outcome supports the null hypothesis of the experiment. Both cultivars showed detectable levels of TRG. A previous study demonstrated that the concentration of TRG in *Glycine max* L. increased fourfold when estimated from dry leaf weight compared with leaf fresh weight under water-deficit stress and interspecific competition stress, respectively (Cho et al. 2002).

This study shows that TRG levels in hemp leaves vary with water availability. This outcome confirms observations in other species that the accumulation of TRG in plant tissues occurs under water-deficit stress (Wood et al. 1996; Pfeiffer et al. 2001). Under water-deficit conditions, TRG levels increased significantly, consistent with a previous study demonstrating that osmotic compatible-solutes help accumulate TRG under water-deficit conditions (Ashihara 2008). Although water-deficit stress increased rapidly, no cultivar-specific differences were detected in TRG levels. This suggests that TRG accumulation may represent a common physiological response to water deficit shared across hemp cultivars. Strong environmental stress can mask genetic differences among cultivars, so the observed response may reflect the severity of water-deficit stress rather than differences in drought tolerance among cultivars (Dodig et al. 2008). Hence, tissue accumulation of TRG could be considered a worthwhile marker of water-deficit stress in hemp (Millar et al. 2011). RWC differed significantly between treatments, confirming the efficacy of the induced water-deficit stress.

Differences in mean RWC showed that, under control conditions, hemp leaves had a higher RWC than under conditions of water-deficit stress. The RWC level was low under water-deficit stress, as observed in the study by Schwartz et al. (2014). However, the present study showed no differences in TRG accumulation or RWC between the two cultivars we tested. Nevertheless, it would be instructive to test additional cultivars, as other hemp cultivars may differ in photoperiod sensitivity under water-deficit stress (Campbell et al. 2019). Likewise, water-deficit conditions can lead to differences in yield loss in different hemp cultivars (Babaei & Ajdanian 2020).

The present study has certain limitations, which indicate the need and open up possibilities for future work. Hemp shows considerable agronomic potential even under water-deficient, stressful conditions, reflecting its tolerance to microbial contamination, potential for growth in contaminated soil, suppression of soil pathogens, and sequestration of soil heavy metals (Sharma et al. 2025). Hence, it is important to identify and study the potential mechanism of accumulation of secondary metabolites, such as TRG, which was not resolved in this study. It remains to be determined whether TRG levels increase under salt and water-deficit stress, when TRG acts as an osmoprotectant, e.g., in *Trigonella foenum-graecum* (Tavangar et al. 2021). Furthermore, it has also not yet been clarified whether there was a biochemical shift in metabolic activity, which may also occur in hemp. The roots and stem fibers of hemp should be further investigated to quantify the accumulation of secondary metabolites, such as TRG, as these metabolites could be exploited for medicinal use (Odieka et al. 2022). In addition, based on a previous study (Da Cunha Leme Filho et al. 2025), we could consider using different plant growth regulators involved in plant growth and development under stress conditions, which may also determine TRG accumulation. Consequently, future research should examine whether the use of plant growth regulators, such as uniconazole, under drought stress conditions may increase levels of compounds involved in osmoregulation. However, it is unclear how changes in endogenous hormone levels under water-deficit stress conditions could affect TRG accumulation in hemp. Therefore, the response of hemp to water-deficit stress is complex and depends on the timing, duration, and severity of the stress, as well as the cultivar type, production purpose, and growing conditions (Sharma et al. 2025). Additionally, since the TRG level did not vary among cultivars, TRG alone cannot be used as an indicator of cultivar water-deficit stress tolerance. Rather, it might be beneficial to combine TRG with growth, yield, water-use efficiency, or root traits for cultivar selection.

CONCLUSIONS

Trigonelline accumulation in hemp leaves was strongly associated with declining relative water content, indicating that TRG responds sensitively to plant water status. Both cultivars demonstrated substantial increases in TRG under water-deficit conditions, suggesting that this response is conserved across genotypes. In particular, water availability had a far greater influence on TRG concentration than genetic background, suggesting that TRG functions primarily as an environmental stress indicator rather than a cultivar-specific trait. These findings position TRG as a robust and genotype-independent biochemical marker for monitoring drought stress in hemp. Its consistent accumulation under water limitation highlights its potential utility for early stress detection and irrigation management in commercial production.

Author contribution statement

SA – A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

AW – A – Research concept and design, C – Data analysis and interpretation, E – Critical revision of the article, F – Final approval of the article

KG – A – Research concept and design, E – Critical revision of the article, F – Final approval of the article

JF– A – Research concept and design, E – Critical revision of the article, F – Final approval of the article

DG – A – Research concept and design, E – Critical revision of the article, F – Final approval of the article

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