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ORIGINAL ARTICLE

Raman-based diagnostics of drought, heat and light-induced stresses in three different varieties of hemp

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Abstract

Main conclusion **Hand-held Raman spectroscopy can be used for highly accurate differentiation between drought, heat and light-triggered stresses in hemp. The differentiation is based on the changes in the biochemistry of plants caused by such stresses.**

Abstract Hemp farming is a rapidly growing industry. This dioecious plant is primarily cultivated for its fibers, seeds, and cannabinoid-rich oils. The yield of these materials can be drastically lowered by many abiotic stresses, such as drought, heat and light. It becomes critically important to develop robust and reliable approaches that can be used to diagnose such abiotic stresses in hemp. In this study, we investigate the accuracy of Raman spectroscopy, an emerging tool within crop monitoring, in the confirmatory identification of drought, heat, and light-induced stresses in three varieties of hemp. Our results showed that mono, double and triple stresses uniquely alter plant biochemistry that results in small spectroscopic changes detected in the Raman spectra acquired from the hemp leaves. These changes could be used for the 80–100% accurate identification of individual abiotic stresses and their combinations in plants. These results demonstrate that a hand-held Raman spectrometer can be used for highly accurate, non-invasive, non-destructive, and label-free diagnostics of hemp stresses directly in the greenhouse or in the field.

Keywords Hemp · Diagnostics · PLS-DA · Raman spectroscopy · Stress

Abbreviations

THC	Tetrahydrocannabinol
CBD	Cannabidiol
RS	Raman spectroscopy
PLS-DA	Partial least squares discriminant analysis
LV	Latent variable
CW	Cherry Wine
JM	Jin Ma
OB	Otto/Boux

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Introduction

Hemp (*Cannabis sativa* sp.) is a dioecious plant that is broadly cultivated for its stem fibers, seeds and cannabinoids, including tetrahydrocannabinol (THC) and cannabidiol (CBD) (Small and Marcus 2002). The fibers are used to fabricate construction materials, textiles, and paper (Rehman et al. 2021), whereas hemp seeds collected from the achenes are commonly used for livestock feed and oil production (Small 2015). Cannabinoids synthesized by hemp buds have a broad spectrum of pharmacological purposes. For instance, CBD can be used to improve sleep quality, suppress anxiety and mitigate symptoms arising from chemotherapy treatment of cancer patients (Small and Marcus 2002; Rehman et al. 2021; Zhelyazkova et al. 2020; Heider et al. 2022; O'Brien 2022). Hemp plants can also uptake high amounts of atmospheric carbon dioxide and remediate contaminated soil through phytoremediation (Adesina et al. 2020).

The legalization of hemp across the U.S. opens the possibility of growing this plant species in geographic areas with low levels of precipitations, higher temperatures and sunlight intensities (Miller 2020). However, hemp optimal vegetation temperatures are within 24–30 °C (Climate and Average Weather Year Round in Winters Texas, United States 2023; Goble 2023). If the temperature rises above 31 °C, severe metabolic and physiological stresses are observed in this plant species (Park et al. 2022). This results in a drastic, 70–80% reduction of the cannabinoid biosynthesis (Park et al. 2022). These devastating effects of harsh weather conditions on cannabinoid production catalyzed the search for analytical approaches that can be used for timely diagnostics of such stresses in hemp (Dey 2022; Industrial hemp faces challenges in Texas 2021).

A growing body of evidence indicates that Raman spectroscopy (RS), an emerging analytical technique, can be used to detect and identify biotic and abiotic stresses in plants. The technique is based on the phenomenon of inelastic light scattering that takes place upon the interactions of photons with the molecules of the sample. In such cases, inelastically scattered photons have lower and higher energies than the energy of the incident light. The change in the photon energy directly depends on the chemical structure of the sample. Biotic and abiotic stresses strongly alter plant biochemistry, which results in substantial changes in the Raman spectra acquired from plant leaves and stalks. For instance, Higgins and co-workers (2022) recently demonstrated that drought and nutritional deficiency in wheat results in a decrease in the concentration of carotenoids (Higgins et al. 2022). Similar changes were also observed upon viral infection and aphid stresses. It was also demonstrated that changes in the concentration of carotenoids in wheat leaves could be non-invasive and non-destructively sensed using RS. Higgins and co-workers (2022) also demonstrated that such spectra could be used for ~ 100% accurate identification of both biotic and abiotic stresses. Similar findings were previously reported by our group for rice. Specifically, Sanchez and co-workers (2020) demonstrated that nitrogen, phosphorus, and potassium deficiency in rice could be detected using RS before the appearance of symptoms associated with these stresses (Sanchez et al. 2020). Similar experimental findings were reported by Rem and Scully groups (Altangerel et al. 2021).

Expanding upon this, we propose to investigate the potential of RS in the diagnostics of heat and drought stresses in three different varieties of hemp. We will also investigate the extent to which RS could be used to detect and identify hemp stress induced by high light intensity. For this, hemp plants were grown in the greenhouse where the stresses were modeled. We acquired RS from these plants and utilized chemometrics to determine the accuracy of stress identification based on the acquired Raman spectra.

Materials and methods

Plants

A total of 120 *Cannabis sativa* L. plants were germinated up to the four-node stage in a greenhouse in College Station, TX. Three different cultivars were used in this experiment. 40 of the plants were Cherry Wine (CW), 40 plants were Jin Ma (JM), and 40 plants were Otto/Boax (OB). These plant varieties are primarily cultivated as a source of CBD. These varieties can be also used for plant fiber. Light stress was induced by increasing the overhead lights from 300 to 700 PAR. Heat stress was induced by placing plants in tents that held a temperature range of 40–43 °C instead of the greenhouse temperature of 32–35 °C. The original unstressed temperature was planned to be around 29.5 °C but ended up being higher due to the weather during the time of inducing stress. The control groups experienced no stress. The plants could experience, one stress or a combination of two or three stresses. There were five plants of every cultivar that experienced the same types of stress, making 15 plants in total for one combination of stress. For example, five Cherry Wine plants experienced only heat stress while 15 plants in total only heat stress. Every combination of stress goes as follows: no stress (control), only heat stress, only light stress, only water stress, heat and light stress, heat and water stress, light and water stress, and heat, light and water stress. There were eight unique groups with 15 plants in each group. Five plants of every group were a unique cultivar. Spectra were taken two, three, six and eight days after stress was induced. The plants were very unhealthy, and the scans were becoming of poor quality by the eighth day, therefore, scanning finished after this day.

Raman spectroscopy

The Agilent Resolve Raman spectrophotometer with an 830-nm laser was used to collect the spectra of the hemp leaves. The following experimental parameters were used to collect all spectra: 495 m W power, 1 s acquisition time and baseline spectral subtraction by device software. We avoided analysis of senescing and just appeared plant leaves. Each plant was scanned twice on two different leaves on the adaxial side. By accumulating ten scans over four time points, roughly 40 spectra were collected for each group that was averaged using MATLAB.

Multivariate statistical analysis

Partial least squares discriminant analysis (PLS-DA) was carried out using MATLAB/PLS_Toolbox (Eigenvector Research Inc.) software for all collected spectra. Spectra was pre-processed by area normalization. Baselining was not done during preprocessing because the handheld Raman automatically baselines every spectra when acquired. Accuracy rates were reported based on the predictions and were made into true prediction rates. A model optimizer was used to find the preprocessing methods that would yield the best accuracy rates. The model optimizer reported the following preprocessing to yield the best results: 1st derivative (Savitzky-Golay) (order:2, window: 15 pt, tails: polynomial interpolation) and then mean centering. Latent variables (LV) were chosen based on CV Classification Error Average, Classification Error Average and root mean square error of cross-validation (RMSECV). LV plots for all developed PLS-DA models are shown in Fig. S1–S84. For all models, Venetian Blinds cross-validation approach was used. Accuracy was calculated by averaging the true prediction rate (TPR) of the model. Consequently, TPRs indicated the accuracy with which the model assigned the spectra to the correct class of data.

Results and discussion

In the Raman spectra collected from hemp cultivars, we observed vibrational bands at 729, 796, 843, 916, 1000, 1047, 1068, 1155, 1185, 1216, 1286, 1326, 1388, 1440, 1525, 1607, 1699, and 1712 cm^{-1}, Fig. 1 and Table 1. These bands can be assigned to carotenoids (1000, 1155–1218 and 1525 cm^{-1}), cellulose (747, 916, and 1047 cm^{-1}) and compounds with carboxylic groups (1712 cm^{-1}) (Fig. 1 and Table 1). (Schulz et al. 2005; Edwards et al. 1997; Devitt et al. 2018; Dou et al. 2021; Adar 2017). It should be noted that vibrational bands at 1326, 1388, and 1440 cm^{-1} originate from CH and CH_2 vibrations and, therefore, cannot be assigned to a particular class of biological molecules (Synytsya et al. 2003; Edwards et al. 1997; Yu et al. 2007). In the spectra acquired from the leaves of hemp plants exposed to the drought, heat, and light-induced stresses, we observed only small changes in the intensities of the discussed above vibrational bands, Fig. 1 and Figures S85–S93. It should be noted that for all three stresses, the major changes were attributed to the intensities of carotenoid vibrations, suggesting that drought, heat, and light induced changes in the concentrations of carotenoids in hemp leaves. Finally, we found that the magnitude of changes in the intensity of carotenoid vibrations was much greater in Cherry Wine and Jin- Ma varieties compared to Otto/Boax, Fig. 1 and Fig. S85–S93. These findings demonstrate that Otto/Boax variety is more resistant to these abiotic stresses than Cherry Wine and Jin- Ma varieties. We also found significant changes in the intensity of 478 cm^{-1} vibration that can be assigned to C–C–O and C–C–C deformations of glycosidic ring of carbohydrates (Farber et al. 2020). Additionally, this band may originate from δ(C–C–C) + τ(C–O) scissoring of C–C–C and out-of-plane bending of C–O vibrations of carbohydrates. Thus, we can conclude that change in the concentration of carbohydrates is linked to heat, drought and light-induced stresses in hemp.

Next, we utilized PLS-DA to investigate the accuracy of identification of drought, heat, and light stresses in the three varieties of hemp. For Cherry Wine, PLS-DA model was able to determine if the plants were under drought (85%), heat (91%) or light stress (92%) with high accuracy. Similar accuracies of the stress identification were found for Jin-Ma plants. Specifically, we were able to identify drought with 96%, heat with 95%, and light with 83% accuracies. Finally, we found that drought in Otto/Boax variety could be diagnosed with 84%, heat with 92%, and light-induced stress with 75%. Based on these results, we can conclude that Raman spectroscopy can be used to detect and identify drought, heat, and light-induced stresses in different varieties of hemp.

One can expect that in the reality of hemp farming, plants may experience more than one stress. Therefore, we investigated whether Raman spectroscopy could be used to detect and identify two and all three stresses simultaneously applied on the hemp plants. Like the discussed above spectroscopic analysis of plants with individual stresses, we observed only small changes in the intensities of vibrational bands that can be assigned to carotenoids in the spectra acquired form hemp exposed to two and three stresses, Fig. 2.

PLS-DA results demonstrated that drought and heat stress could be identified on Cherry Wine with 96%, whereas heat and light and light and drought stresses could be diagnosed with 87% and 92% on this variety, respectively. Our results also showed that all three stresses could be confirmatory identified with 95% accuracy. Similar accuracies of the identification of dual stresses were observed for Jin-Ma variety. Specifically, drought and heat stress could be identified with 100% accuracy, whereas heat and light and light and drought with 100% and 89% accuracies, respectively. Finally, we were able to correctly predict the effect of all three stresses with 98% accuracy. On Otto/Boax plants, RS enabled 94% accurate identification of drought and heat stresses, whereas

Fig. 1 Averaged Raman spectra acquired from Cherry Wine (**A**), Jin- Ma (**B**) and Otto/Boax (**C**) varieties of hemp exposed to the drought (DNN), heat (NHN), and light (NNL) stresses, as well as control plants (NNN). Spectra are normalized on 1440 cm^{-1}

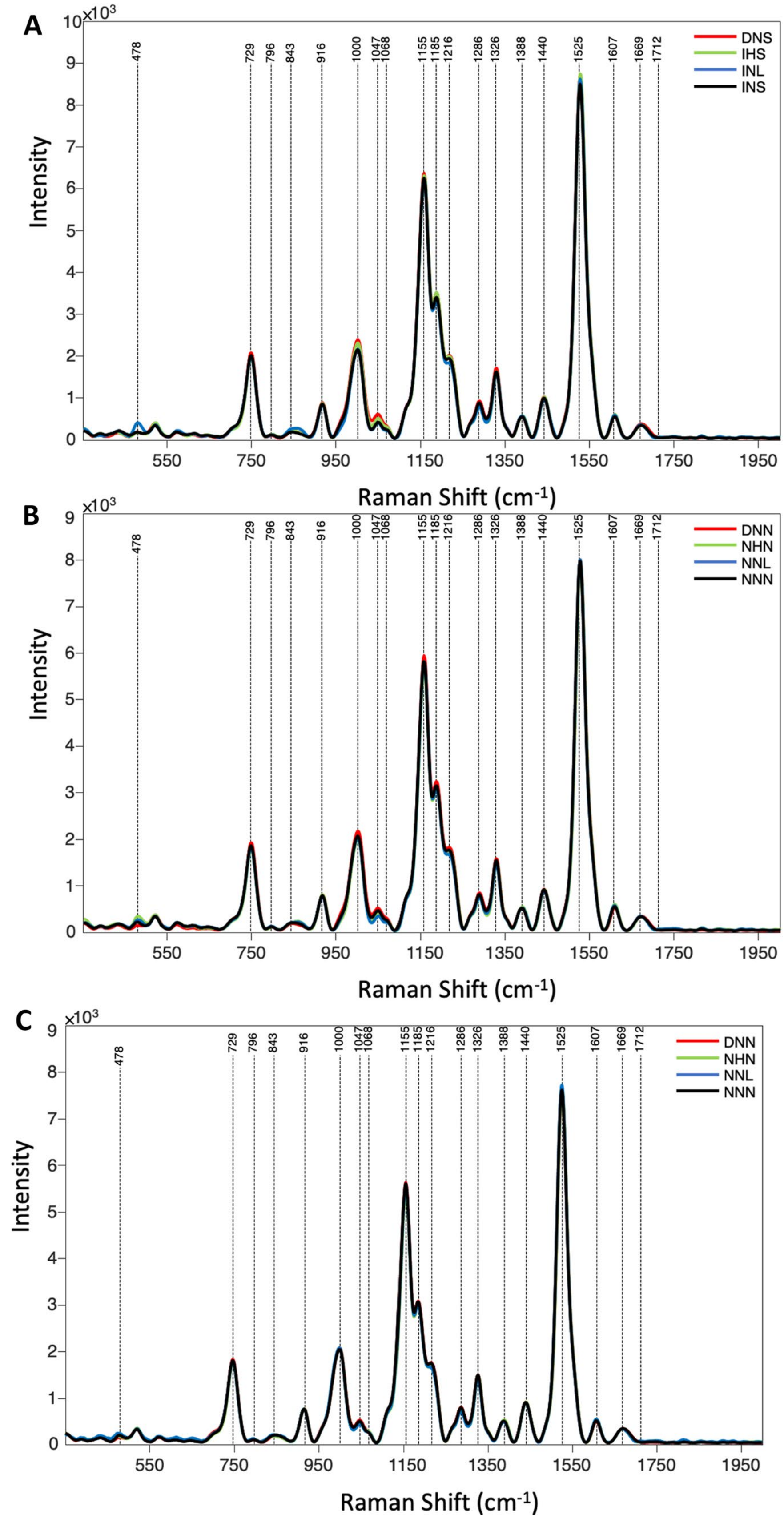

Table 1 PLS-DA results for identification of drought, heat and light stresses in Cherry Wine, Jin-Ma and Otto/Boax varieties of hemp

Variety	Stress	Accuracy, %	Variety	Stress	Accuracy, %	Variety	Stress	Accuracy, %
Cherry wine	Drought	85% (LV = 3)	Jin- Ma	Drought	96% (LV = 5)	Otto/Boax	Drought	84% (LV = 3)
	Heat	91% (LV = 3)		Heat	95% (LV = 3)		Heat	92% (LV = 4)
	Light	92% (LV = 4)		Light	83% (LV = 2)		Light	75% (LV = 2)

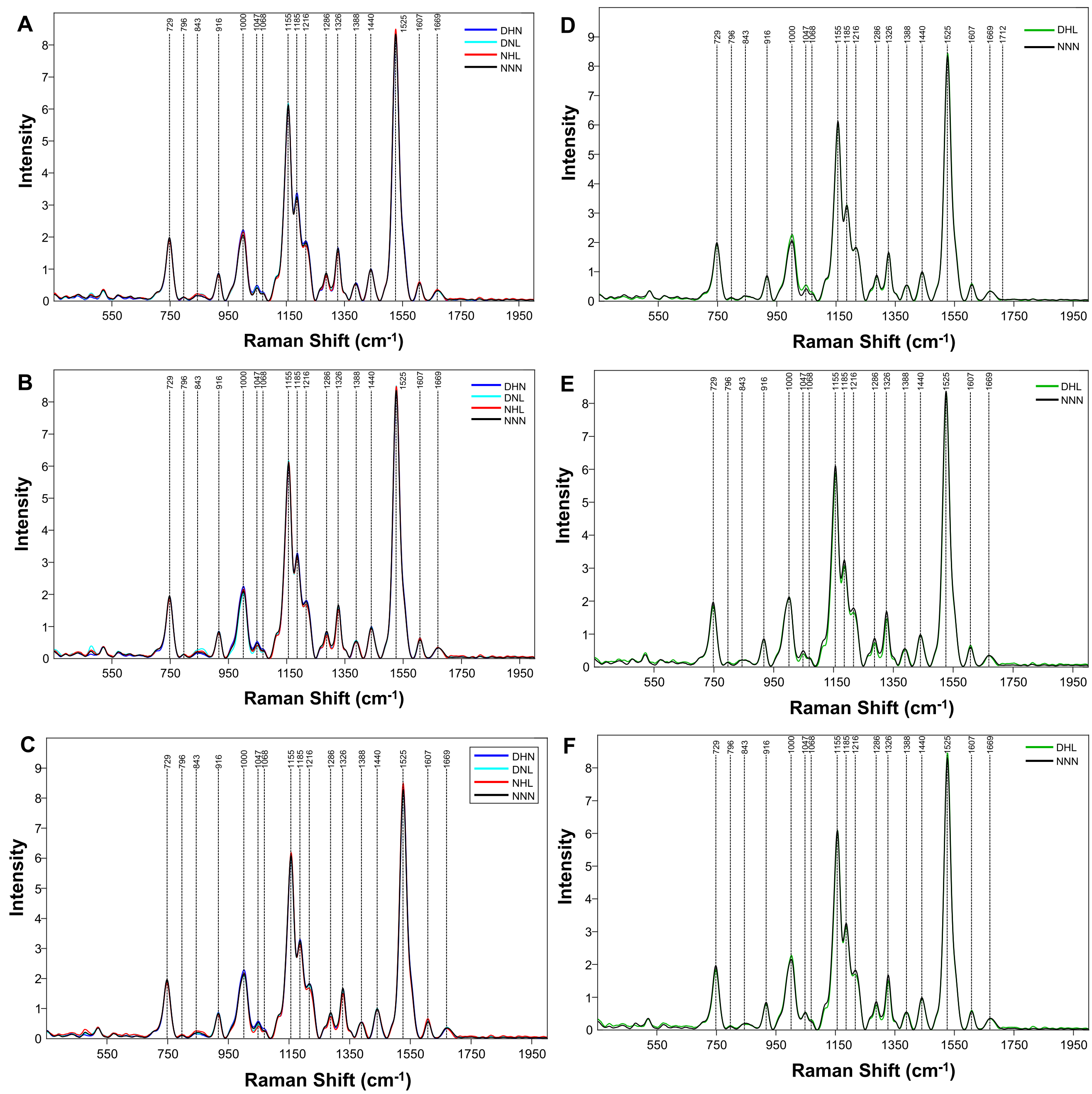

Fig. 2 Raman spectra acquired from Cherry Wine (**A** and **D**), Jin-Ma (**B** and **E**) and Otto/Boax (**C** and **F**) varieties of hemp exposed to the drought and heat (DHN), drought and light (DNL), and heat and light (NHL) stresses (A-C), as well as all three (DHL) stresses (D-F). Spectra acquired from the control plants are labeled (NNN)

Table 2 PLS-DA results for identification of dual drought-heat, heat-light and light-drought stresses, as well as all three stresses in Cherry Wine, Jin-Ma and Otto/Boax varieties of hemp

Variety	Stress	Accuracy, %	Variety	Stress	Accuracy, %	Variety	Stress	Accuracy, %
Cherry wine	Drought and heat	96% (LV = 2)	Jin- Ma	Drought and heat	100% (LV = 2)	Otto/Boax	Drought and heat	94% (LV = 4)
	Heat and light	87% (LV = 4)		Heat and light	100% (LV = 2)		Heat and light	91% (LV = 4)
	Light and drought	92% (LV = 2)		Light and drought	89% (LV = 3)		Light and drought	81% (LV = 2)
	Drought, heat and light	95% (LV = 3)		Drought, heat and light	98% (LV = 4)		Drought, heat and light	95% (LV = 2)

Table 3 PLS-DA results for identification of all modelled stresses in Cherry Wine plants

	Drought and heat	Light and drought	Heat and light	Drought	Heat	Light
Drought, heat and light	87.6% (LV = 4)	91% (LV = 4)	78% (LV = 3)	90% (LV = 5)	80% (LV = 4)	88% (LV = 5)
Drought and heat		91% (LV = 4)	93% (LV = 3)	93% (LV = 4)	65% (LV = 2)	85% (LV = 3)
Light and drought			72% (LV = 3)	90% (LV = 3)	94% (LV = 3)	72% (LV = 2)
Heat and light				88% (LV = 4)	93% (LV = 4)	82% (LV = 4)
Drought					90% (LV = 2)	89% (LV = 4)
Heat						79% (LV = 3)

The table reports the results of cross-comparison of different classes of data described in columns and rows

Table 4 PLS-DA results for identification of all modelled stresses in Jin-Ma plants

	Drought and heat	Light and drought	Heat and light	Drought	Heat	Light
Drought, heat and light	85% (LV = 1)	94% (LV = 3)	76% (LV = 2)	95% (LV = 4)	84% (LV = 2)	83% (LV = 4)
Drought and heat		93% (LV = 2)	96% (LV = 2)	98% (LV = 4)	80% (LV = 3)	94% (LV = 5)
Light and drought			76% (LV = 3)	96% (LV = 2)	82% (LV = 3)	67% (LV = 3)
Heat and light				94% (LV = 4)	90% (LV = 2)	75% (LV = 4)
Drought					93% (LV = 2)	94% (LV = 6)
Heat						75% (LV = 2)

The table reports the results of cross-comparison of different classes of data described in columns and rows

Table 5 PLS-DA results for identification of all modelled stresses in Otto/Boax plants

	Drought and heat	Light and drought	Heat and light	Drought	Heat	Light
Drought, heat and light	93% (LV = 2)	80% (LV = 3)	80% (LV = 2)	90% (LV = 3)	87% (LV = 3)	84% (LV = 4)
Drought and heat		95% (LV = 3)	96% (LV = 4)	92% (LV = 5)	67% (LV = 1)	79% (LV = 3)
Light and drought			84% (LV = 3)	86% (LV = 2)	87% (LV = 3)	69% (LV = 5)
Heat and light				87% (LV = 3)	93% (LV = 4)	72% (LV = 2)
Drought					88% (LV = 2)	91% (LV = 5)
Heat						77% (LV = 2)

The table reports the results of cross-comparison of different classes of data described in columns and rows

heat and light and light and drought could be detected with 91% and 81% accuracies, respectively. RS also enabled 95% accurate identification of the effect of all three stresses in this variety, Table 2.

Finally, we want to investigate the robustness of the discussed above chemometric models and investigate the accuracy with which all modelled stresses can be differentiated from each other, Table 3, 4 and 5. In these models, we cross-compared different stresses with each other without the direct comparison to the control. Our results showed that in most cases, above 90% accurate differentiation between the stresses was possible. We found that on average for all three varieties, only 4–9 stresses could be identified with < 80% accuracy.

Conclusion

Our results show that Raman spectroscopy could be used for highly accurate (~92%, on average) diagnostics of drought, heat, and light-induced stresses in all three varieties of hemp. Such diagnostics is non-invasive, non-destructive, and label-free. Furthermore, utilization of a hand-held Raman spectrometer. enables direct sensing of the plant health in the greenhouse setting.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00425-023-04299-6.

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Author contribution MS and KM performed the experiment, collected and analyzed spectra. MS performed PLS-DA; RJ and DK conceptualized the idea, supervised the project, wrote the manuscript and administrated the work.

Data availability The data will be made available on reasonable request to the corresponding author.

Declarations

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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