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Optimizing in vitro germination of primed industrial hemp (*Cannabis sativa* L.) seeds

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Priming edilmiş endüstriyel kenevir (*Cannabis sativa* L.) tohumlarının in vitro çimlenmesinin optimize edilmesi

Abstract: Germination under in vitro conditions for industrial hemp (*Cannabis sativa* L., *Cannabaceae*) is highly significant for the application of biotechnological tools like genome editing. Therefore, seeds were surface sterilized followed by priming with hydrogen peroxide (H_2O_2) and sterile distilled water (dH_2O) for 24, 48, and 72 h. The primed seeds were inoculated on the Murashige and Skoog (MS) medium enriched with 1.0 mg/L benzyl amino purine (BAP) and 200 mg/L antibiotics. Exposing seeds to H_2O_2 was superior and 100% germination was observed. Whereas hydropriming resulted in 30-52.5% germination with a maximum of 48h priming time. The results on shoot counts revealed a maximum of 1.98 shoots from the combination of 3.0% H_2O_2 and 72h priming time. The results were also analyzed by constructing different statistical plots like box plots, normal plots, contour plots, and surface plots. The normal plots exhibited the significance of H_2O_2 concentration on both output variables. Whereas contour and surface plots classified the output data into different sub-groups and confirmed the results.

Key words: Germination, industrial hemp, in vitro, priming, statistical analysis

Özet: Endüstriyel kenevir (*Cannabis sativa* L., *Cannabaceae*) bitkisinin in vitro çimlenmesi, genom düzenleme gibi biyoteknolojik araçların uygulanması için oldukça önemlidir. Bu nedenle, tohumlar yüzey sterilize edildikten sonra 24, 48 ve 72 saat boyunca hidrojen peroksit (H_2O_2) ve steril distile su (dH_2O) ile priming uygulama yapılmıştır. Daha sonra tohumlar, 1.0 mg/L BAP ve 200 mg/L antibiyotik ile zenginleştirilmiş Murashige ve Skoog (MS) ortamı üzerinde kültüre alınmıştır. Tohumların H_2O_2 ile yapılan priming sonucunda daha iyi sonuç verdiğini ve %100 çimlenme olduğu gözlemlenmiştir. Buna karşı hidropriming ile yapılan çalışmada 30-52,5 çimlenme gözlenirken, en iyi sonucun 48 saatlik priming sonucu olduğu kaydedilmiştir. Sürgün sayılarına bakıldığında en fazla 1,98 sürgün, %3,0 H_2O_2 ve 72 saatlik priming süresi kombinasyonundan elde edilmiştir. Sonuçlar ayrıca kutu grafiği, normal grafikler, kontur grafikleri ve yüzey grafikleri gibi farklı istatistiksel grafikler oluşturularak da analiz edilmiştir. Normal grafikler, H_2O_2 konsantrasyonunun her iki çıktı değişkenindeki önemini açıkça göstermiştir. Kontur ve yüzey grafikleri ise çıktı verilerini farklı alt gruplara ayırmış ve sonuçları doğrulanmıştır.

Anahtar Kelimeler: Çimlenme, endüstriyel kenevir, in vitro, priming, istatistiksel analiz

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1. Introduction

Industrial hemp (*Cannabis sativa* L., *Cannabaceae*) is one of the most significant and commercially grown crops of the World, due to its multiple uses in different industries ranging from pharmaceutical to the fiber industry (Salentijn et al., 2014; Tremlová et al., 2021). The commercially grown area of industrial hemp is increasing significantly with an estimated compound annual growth rate (CAGR) of 16.2% up to 2028 (Aasim et al., 2022). The demand for developing new cultivars with elite characteristics (Chandran et al., 2020) enforces researchers to employ biotechnological tools like plant tissue culture. The in vitro germination followed by the seedling establishment is simultaneously a physiological process (Ventura et al., 2012), regulated by both internal and external stimuli. Exposing seeds to any external stimuli within a specific time may lead to the manipulation of a whole germination process (Paparella et al., 2015). The priming is the exogenous appliation of a physical or chemical stimulus to the seeds, and to date, various priming techniques have been developed and employed successfully for different crops under different culture conditions (Salah et al., 2015).

Germination of cannabis seeds under in vitro conditions is a major issue and needs the use of some external stimuli like water or H_2O_2. Hydrogen peroxide (H_2O_2) is an oxidant (Sevilgen and Velioğlu, 2009), that generate in response to stresses in plants (Karataş et al., 2015). The significance of H_2O_2 for plants is relatively high irrespective of complicated relationships, which often may lead to oxidative damage leading to cell death (Abbas et al., 2011; Hossain et al., 2015; Zhang et al., 2015). Therefore, automation of the optimum dose of H_2O_2 and treatment time is important. Hydropriming is another and most promising technique used in seed germination. In this study, the surface sterilized seeds of industrial hemp were treated with H_2O_2 and sterile distilled water (dH_2O) for three different treatment times to check the impact on germination and shoot counts.

2. Materials and Method

The cultivated industrial hemp population "Narlısaray" was procured from Gokhoyuk Agricultural Management Directorate, Amasya, Türkiye.

The healthy seeds were selected manually and the damaged seeds were discarded. The seeds were surface sterilized following Aasim et al., (2022). The seeds were treated with ethanol (70% for 3 min), followed by treatment with $HgCl_2$ (0.10% for 10 min). Thereafter, distilled water was used for rinsing the seeds and this process was repeated three times for 5 min each for removing the traces of the disinfectant.

The surface sterilized seeds were exposed to 2.0% and 3.0% v/v H_2O_2 (34.5–36.5% H2O2 - Sigma-Aldrich) for 24, 48, and 72 h. The same treatment time was also used for hydropriming (Control) of industrial hemp seeds. After the respective treatment time, the seeds were transferred to the culture medium for germination.

The culture medium used in this study comprised of Murashige and Skoog (MS) medium (Murashige and Skoog 1962), 30 g/L sucrose, and 6.5% agar as a gelling agent. The pH was automated with 1.0 N HCl/NaOH to approximately 5.8 after adding 1.0 mg/L benzylamino purine (BAP). To overcome the issue of endogenic bacteria, 200 mg/L Sulcid (ampicillin and sulbactam) was also added to the culture medium after autoclaving the medium. Autoclaving of the culture medium was performed as a standard procedure employed (15 min, 121 ◦C, 1.5 atm pressure). The basal medium containing primed seeds were placed under White Light-emitting Diodes (LEDs) with temperature and light photoperiod automated at 24 ± 1 ◦C and 16 h respectively.

The experiment was performed in 4 replicates containing 10 seeds per replicate. The data regarding in vitro germination was tabulated after 21 days of culture. Whereas, shoot counts were recorded after 6 weeks of culture. The data were analyzed using two different statistical programs for a better presentation of data. The ANOVA, regression equation, and normal plots were analyzed using Minitab statistical software. The difference among the treatments was analyzed with Tukey's B. The contour and surface plots were developed with the aid of a design expert program.

3. Results

Application of H_2O_2 concentration (C) and treatment time (T) significantly affected the in vitro germination (%) and shoot counts of surface sterilized seeds of industrial hemp. The results revealed that concentration and treatment time did not affect the germination, percentage (100%). Exposing seeds to water (control treatment-hydropriming) for 24 to 72 h yielded 30.0-52.50% germination with a maximum from 48 h treatment time. The results on shoot counts ranged 1.05-1.98. The maximum shoot counts were recorded from 3.0% H_2O_2 for 72 h treatment time. On the contrary, minimum shoot counts were attributed to seeds used in control treatment for 72 h (Table 1). The results were further illustrated by constructing a box plot analysis of both output parameters (Fig. 1a,b).

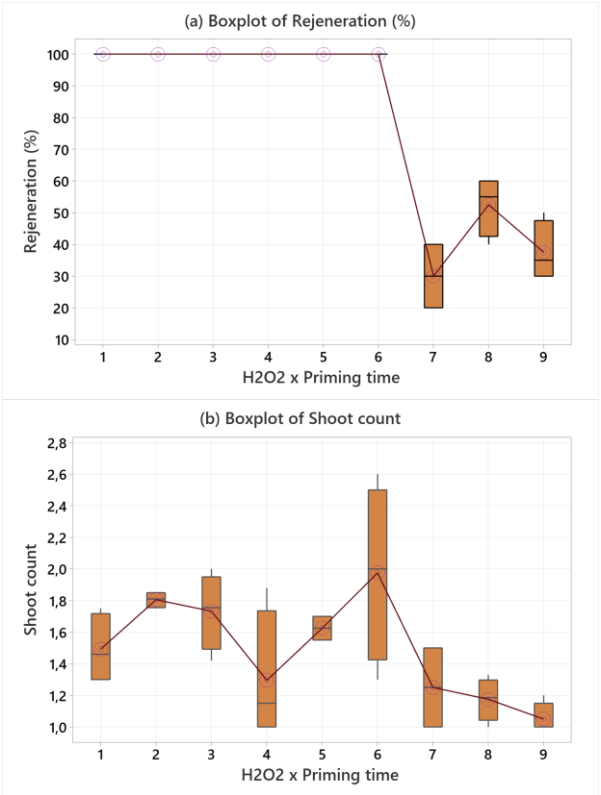

Figure 1. Box plot analysis of in vitro germination and shoot counts in response to priming agent concentration and treatment time.

Table 1. Impact of C × T of H_2O_2 and dH_2O on the germination (in vitro) of industrial hemp (*Cannabis sativa* L.)

H_2O_2 (%)	Time (h)	Germination (%)			Shoot counts		
		Mean	StDev	95% CI	Mean	StDev	95% CI
2	24	100.0A	0.0	(93.9; 106.1)	1.49ABC	0.229	(1.204; 1.781)
2	48	100.0A	0.0	(93.9; 106.1)	1.81AB	0.0526	(1.5161; 2.0939)
2	72	100.0A	0.0	(93.9; 106.1)	1.73AB	0.241	(1.444; 2.021)
3	24	100.0A	0.0	(93.9; 106.1)	1.30BC	0.415	(1.006; 1.584)
3	48	100.0A	0.0	(93.9; 106.1)	1.63ABC	0.0866	(1.3361; 1.9139)
3	72	100.0A	0.0	(93.9; 106.1)	1.98A	0.556	(1.686; 2.264)
0	24	30.00C	11.55	(23.91; 36.09)	1.25BC	0.289	(0.961; 1.539)
0	48	52.50B	9.57	(46.41; 58.59)	1.18BC	0.1358	(0.8861; 1.4639)
0	72	37.50C	9.57	(31.41; 43.59)	1.05C	0.1000	(0.7611; 1.3389)

3.1. Normal plot analysis

Normal plot analysis was performed to check the significant level of input variables of concentration, treatment time, and interaction of C × T time. Results revealed that priming agent concentration exhibited a positive impact on in vitro germination only. The impact of treatment time and C × T remained non significant (Fig. 2a,b). Whereas, the priming agent concentration and C × T exhibited a positive impact on shoot count compared to priming time which remained non significant (Table 1).

3.2. Contour and surface plot analysis

The results were further analyzed with the aid of 2-D contour plots (Fig. 3a,b), and 3-D surface plots (Fig. 4a,b). The analysis of contour plots revealed the distribution of germination into 20-100% and shoot counts as 0-2.0, expressed with a different color. The results of contour plots were further confirmed with surface plots.

4. Discussions

Germination is a complex process and is regulated by several internal and external stimuli (Wojtyla et al., 2016). Some plant seeds are known as recalcitrant nature due to difficulties in germination under specific conditions. Plant tissue culture offers a novel technique to germinate seeds more efficiently due to controlled conditions (Karataş et al., 2016). In vitro germination of industrial hemp seeds is relatively difficult and needs some stimuli or some treatments to enhance germination.

H_2O_2 is the potent priming agent for enhancing in vitro seed germination of industrial hemp (Sorokin et al., 2021; Aasim et al., 2022). The results confirmed that priming seeds with

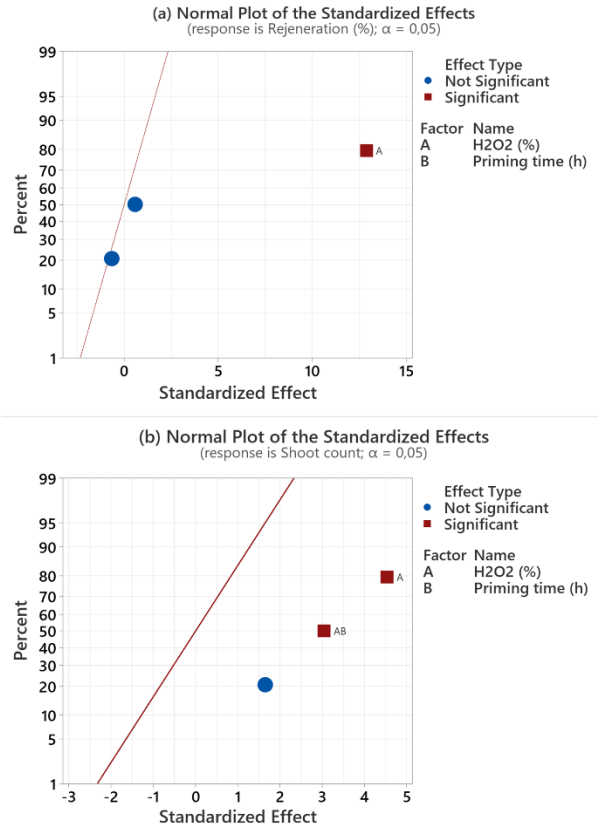

Figure 2. Normal plot analysis of in vitro germination and shoot counts in response to priming agent concentration and treatment time.

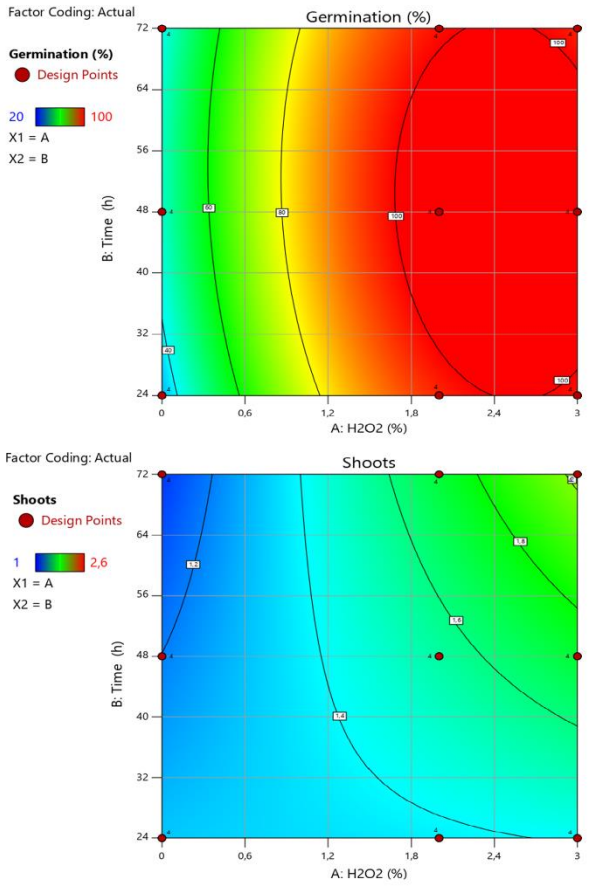

Figure 3. Contour plot analysis of in vitro germination and shoot counts in response to priming agent concentration and treatment time

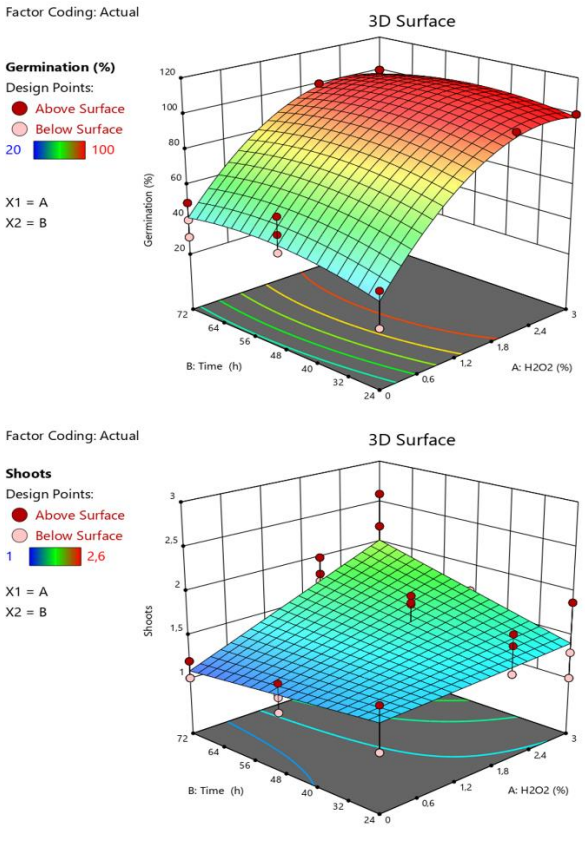

Figure 4. Surface plot analysis of in vitro germination and shoot counts in response to priming agent concentration and treatment time.

H_2O_2 yielded more germination and shoot count compared to hydropriming. The results further confirmed that treatment time also affected both output parameters but exhibited the opposite impact with the priming agent. The positive impact of H_2O_2 improved kinetics of water uptake ending up in higher seed germination (Anawar et al., 2011). However, the treatment time and concentration are the critical factors responsible for a positive impact on plant growth and development (Aasim et al., 2022). The results were also analyzed by box plot which exhibited great variation for germination in response to control treatment. Whereas, variable variation responses among treatments were observed for shoot counts.

Normal plot analysis is a powerful tool to express the positive or negative impact of input variables on the concerning output parameter. The results revealed that the priming agent and its concentration is more significant than other treatment time for both output parameters used in this study. Whereas, treatment time has no impact and remained non significant for both output variables. The normal plot analysis distributes the data on the graph based on significant and non significant level, expressed with different colors and shape inclined on the line (Ramazan and Uğur, 2014; Katirci, 2015). Results of normal plots exhibited the significant factors with a level of input variables. The use of normal plots has been used for investigating the impact of BAP-IBA level on in vitro regeneration of sorghum plant (Aasim et al., 2023).

Conclusion

Germination is a complex physiological and biological process and can be enhanced by employing priming techniques. H_2O_2 is a potent priming agent, and priming seeds with H_2O_2. This proved to be more efficient for high germination and shoot count compared to hydropriming. On the other hand, treatment time had a minimum impact on germination. Results analyzed through normal, contour, and surface plots precisely confirmed the results.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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References

Aasim M, Ali SA, Altaf MT, Ali E, Nadeem MA, Baloch FS (2023). Artificial neural network and decision tree facilitated prediction and validation of cytokinin-auxin induced in vitro organogenesis of sorghum (*Sorghum bicolor* L.). Plant Cell, Tissue and Organ Culture (PCTOC) 1-14.

Aasim M, Katırcı R, Akgur O,Yildirim B, Mustafa Z, Nadeem MA,Yılmaz G (2022). Machine learning (ML) algorithms and artificial neural network for optimizing in vitro germination and growth indices of industrial hemp *(Cannabis sativa L.).* Industrial Crops and Products 181: 114801.

Abass SM, Mohamed HI (2011). Alleviation of adverse effects of drought stress on common bean (*Phaseolus vulgaris* L.) by exogenous application of hydrogen peroxide. Bangladesh Journal of Botany 41: 75-83.

Anawar H, Canha N, Freitas M, Santa Regina I, Garcia-Sanchez A (2011). Effects of Different Drying Processes on the Concentrations of Metals and Metalloids in Plant Materials. J Radioanal Nucl Chem 289: 29-34.

Chandran H, Meena M, Barupal T, Sharma K (2020).Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. Biotechnology Reports 26: e00450.

Hossain MA, Bhattacharjee S, Armin SM, Qian P, Xin W, Li HY (2015). Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. Frontiers in Plant Science 6: 420.

Karatas M, Dogan M, Emsen B, Aasim M (2015). Determination of in vitro free radical scavenging activities of various extracts from in vitro propagated (*Ceratophyllum demersum L.*) Fresenius Environmental Bulletin 24(9a): 2946-2952.

Karataş M, Aasim M, Dazkirli M (2016). Influence of light-emitting diodes and benzylaminopurin on adventitious shoot regeneration of water hyssop (*Bacopa monnieri* (L.) Pennell) in vitro. Archives of Biological Sciences 68(3): 501-508.

Katirci R (2015). Statistical approach to optimizing a Zn-Ni bath containing ed and tea. Surface Review and Letters 22: 1550015.

Ramazan K, Uğur Y (2014). Statistical Studies of Zn-Ni Alloy Coatings Using Non-Cyanide Alkaline Baths Containing Polyethyleneimine Complexing Agents. Trans IMF 92: 245-252.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Paparella S, Araújo SS, Rossi G, Wijayasinghe M, Carbonera D, Balestrazzi A (2015). Seed priming: state of the art and new perspectives. Plant Cell Reports 34: 1281-1293.

Salah SM, Yajing G, Dongdong C, Jie L, Aamir N, Qijuan H (2015). Seed priming with polyethylene glycol regulating the physiological and molecular mechanism in rice (*Oryza sativa* L.) under nano-ZnO stress. Scientific Reports 5: 14278.

Salentijn EMJ, Zhang Q, Amaducci S, Yang M, Trindade LM (2014). New developments in fiber hemp (*Cannabis sativa* L.) breeding. Industrial Crops and Products 68: 32-41.

Sorokin A, adav NS, Gaudet D, Kovalchuk I (2021). Development and standardization of rapid and efficient seed germination protocol for (*Cannabis sativa*) Bio-protocol 11(1): e3875-e3875.

Tremlová B, Mikulášková HK, Hajduchová K, Jancikova S, Kaczorová D, Ćavar Zeljković S, Dordevic D (2021). Influence of technological maturity on the secondary metabolites of hemp concentrate *(Cannabis sativa* L.*).* Foods 10(6): 1418.

Ventura L, Dona M, Macovei A, Carbonera D, Buttafava A, Mondoni A (2012). Understanding the molecular pathways associated with seed vigor. Plant Physiology and Biochemistry 60: 196-206.

WojtylaŁ, Lechowska K, Kubala S, Garnczarska M (2016). Different modes of hydrogen peroxide action during seed germination. Frontiers in plant science 7: 66.

Zhang W, Wang XM, Rong FAN, Yin GX, WANG K, Du LP, Xiao LL, Ye XG (2015). Effects of inter-culture, arabinogalactan proteins, and hydrogen peroxide on the plant regeneration of wheat immature embryos. Journal of Integrative Agriculture 14(1): 11-19.