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Preparation of Plant-derived Smoke for Stimulating Seed Germination and Quantification of Karrikins Using High Performance Liquid Chromatography

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ABSTRACT

Smoke water (SW) is produced naturally or artificially from burning plant material. It provides the germination of the seeds of many plants and accelerates the growth and development of the plant and is also used in many fields of plant science. SW preparation is a relatively easy and inexpensive method, but a standard method for its preparation has yet to be developed. For this reason, our research aims to develop a low-cost efficient method to produce SW, to standardize it and to measure the amount of the main active biomolecule karrikin (KAR_1) by HPLC. We also aimed to test and compare the best working concentration of SW and commercially available KAR_1 on apricot (*Prunus armeniaca* L.) seeds. The SWs were diluted to 1:100, 1:500, 1:1000, 1:5000 and 1:10000 ratios and KAR_1 to 0.01 µM, 0.1 µM, 1 µM, 5 µM and 10 µM concentrations. In terms of germination, it was determined that the use of 1:1000 (60%) concentration in the SW group and 1 µM (72%) concentration in the KAR_1 group was appropriate. This is the first research in which a standard method was developed for obtaining SW. We believe this study will be a guide to researchers who study with SW, since we obtained the most concentrated KAR_1 according to the literature.

Keywords: Smoke water, KAR_1, Karrikinolide, *Prunus armeniaca*, Plant growth regulator

1. Introduction

Mediterranean-type ecosystems in the world are located on the Pacific coasts of Chile and California, in the western and southern parts of Australia, in the Cape region of South Africa and in the Mediterranean basin (Türkan et al. 1985; Beeby & Brennan 1997). Since fire is a phenomenon that shapes vegetation in Mediterranean-type ecosystems, it has a significant importance in the evolution of plants that spread in these ecosystems, and as a result of natural selection, they have developed some adaptation mechanisms to survive. These adaptation mechanisms are resistant tree bark formation, re-shooting after fire, bradispory that holding the seeds such as inside cones and fruits and releasing the seeds after, fire-induced flowering, easy flammability, early reproductive initiation, and fire-induced germination (Tavşanoğlu & Gürkan 2004). Germination induced by fire occurs in two ways. In species with fire-induced germination, dormancy is provided by the seed coat (testa), which prevents the exchange of water and gases. Temperature shock cracks or melts this hard outer cover (testa), allowing water to pass through and germination is occurred (Christensen 1985; Keeley 1995). The second occurs chemically by the presence of burnt wood in the environment and by means of smoke (Keeley et al. 1985; Keeley & Pizzorno 1986; Keeley & Fotheringham 1997; Keeley & Fotheringham 1998). It has been reported by De Lange & Boucher (1990) that plant-derived smoke promotes seed germination more than temperature. Studies have shown that plant-derived smoke positively affects the seed germination of 1200 plant species from 80 different genera, including Arabidopsis (Chiwocha et al. 2009). It has been found that not only the smoke generated as a result of forest fires promotes seed germination, but also the smoke produced under laboratory conditions promotes seed germination. It has been observed that the smoke obtained by burning the *Themeda triandra* Forssk. plant promotes germination in dormant seeds (Baxter et al. 1994). When dry or wet plant material is burned (active substances are formed at 160-200 °C), water-soluble volatile compounds that are formed evaporate at high temperatures, and when dissolved in water, they promote the germination of seeds of many species. Besides this germination effect, smoke water (SW) also promotes seedling growth, shoot branching, root formation, flowering, and tolerance in situations of abiotic stress (Brown & Van Staden 1997; De Cuyper et al. 2017). Seventy one compounds have been identified in the active part of plant-derived smoke (Baldwin et al. 1994) from these compounds, butenolides, nitrogen oxides and cyanohydrins were found to have germination- promoting properties (Nelson et al. 2012). These compounds are water-soluble, can maintain their structure for a long time, are heat-resistant and have high activity at low concentrations (Baldwin et al. 1994; Van Staden et al. 2000). Flematti et al. (2004), separated the SW into fractions by liquid chromatography and used each fraction for seed germination test and thus determined the active compound in the SW. This compound is a special type of lactone with the systematic name 3-methyl-2H-furo[2,3-c]pyran-2-one containing only C, H and O, and because of this property, it resembles strigolactones (Flematti et al. 2015). This compound is a substance belonging to the group of karrikins (KAR) in chemical structure and was named karrikinolide. KAR are abbreviated as KAR and are numbered according to their identification in smoke (Figure 1). Six KAR have been discovered so far. These have been named KAR_1, KAR_2, KAR_3, KAR_4, KAR_5 and KAR_6, but KAR_1 is generally the most abundant in smoke and most active in seed germination (Chiwocha et al. 2009; Nelson et al. 2012; Flematti et al. 2015; De Cuyper et al. 2017). There are many studies that SW and KAR germinate seeds, and all studies show that these substances increase the germination rate (Baxter & Van Staden 1994; Tavşanoğlu 2011; Çatav et al. 2012; Chumpookam et al. 2012; Çatav et al. 2014; Kazancı 2014; Kochanek et al. 2016; Tavsanoğlu et al. 2017; Çatav et al. 2018a). KAR stimulate the formation of a new flora in the burned area by promoting the germination of dormant seed by breaking dormancy, and it has also been reported as a result of various studies that it stimulates the germination of parasitic plants such as Striga and Orobanche (De Cuyper et al. 2017).

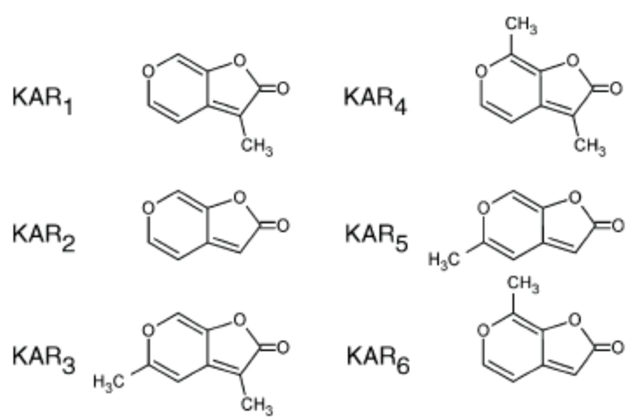

Figure 1- The karrikin family occurring in smoke water (Hrdlička et al. 2019)

Plant-derived smoke compounds cause many changes in seeds, from changes in seed sensitivity to phytohormones and light requirements, testa morphology and permeability properties (Chiwocha et al. 2009). Since KAR_1 stimulates the germination of many species and acts at very low concentrations (<1 ppb or 1 nM), it is hypothesized that it may act by affecting the production or metabolism of other phytohormones. The phytohormones gibberellic acid (GA) and abscisic acid (ABA) are widely accepted as essential endogenous regulators, playing mostly antagonistic roles in plant growth processes and environmental responses. Auxin, one of the phytohormones, is effective in elongation, cell, and tissue differentiation in plants. Of the natural auxins, indole-3-acetic acid (IAA) is the richest auxin in plants and the only endogenous molecule that directly activates auxin signals (Xu et al. 2021). The signaling pathway of protein degradation from phytohormones (GA, IAA and ABA) mainly includes the phytohormone receptor (GID1 for GA, TIR1/AFB for IAA and PYR/PYL/PCAR for ABA), F-box protein (SLY/GID2 for GA, TIR1 for IAA and PP2Cs for ABA), transcription repressor protein (DELLA for GA, AUX/IAA for IAA and SnRK2s for ABA), and transcription factor (GAMYB for GA, ARF for IAA and TFs for ABA). Transcription repressor proteins can interact with various transcription factors and change their activity. When the receptor detects and binds to phytohormones (GA, IAA, and ABA), its structure changes. The N-terminal of the receptor wraps the phytohormone and interacts with the transcription repressor protein. Then, the phytohormone-receptor-transcription repressor protein complex binds to the F-box protein and is subsequently degraded by ubiquitination of the transcription repressor protein (leading to disinhibition of transcription repressor protein and activating phytohormones response genes). The released transcriptional factors then mediate the expression of genes (*EXP2* for GA, *IAA1* for IAA and *ABI3* for ABA) that cause the physiological and morphological responses of seeds or plants to KARs (Figure 2) (Sirko et al. 2021, Xu et al. 2021).

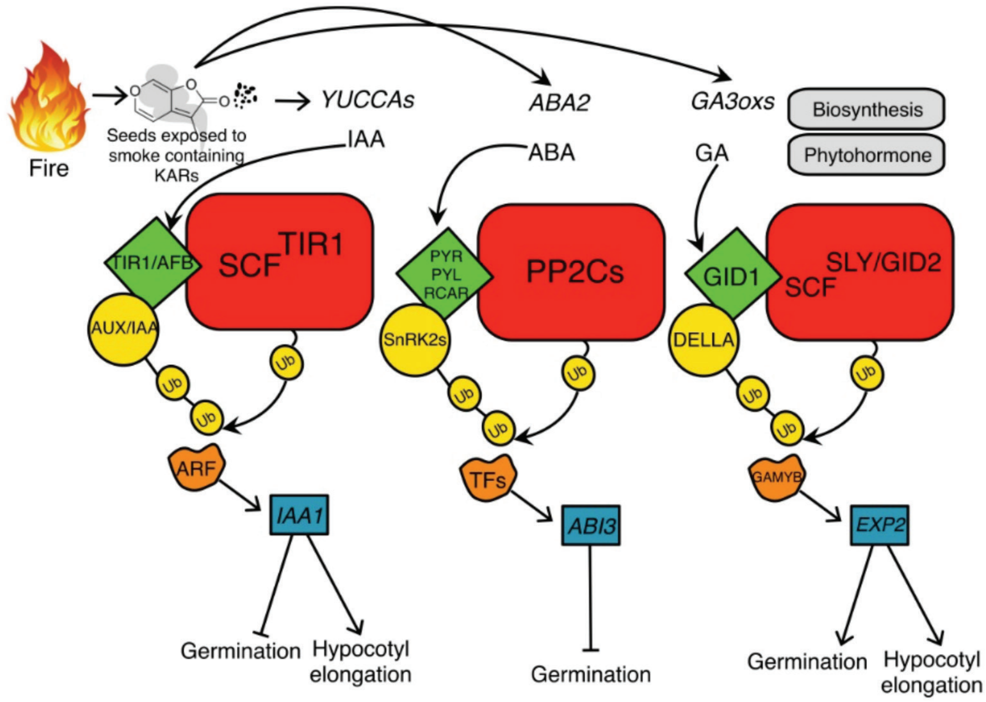

Figure 2- Karrikins regulate seed germination and hypocotyl elongation by affecting phytohormones
IAA inhibits germination and promotes hypocotyl elongation. ABA inhibits seed germination. GA promotes both seed germination and hypocotyl elongation

There are many studies that SW and KAR_1 affect GA, ABA and IAA metabolism (Grossmann 1990; Van Staden et al. 1995; Bewley 1997; Kucera et al. 2005; Merritt et al. 2006; Daws et al. 2007; Stevens et al. 2007; Commander et al. 2009; Nelson et al. 2009). However, this mechanism is still not fully known. KARs are water-soluble substances. KARs have seed germination promoting activity at very low concentrations, usually below 10^{-9} mol L^{-1} (Light et al. 2009; Nelson et al. 2012). However, it is known that SW tends to have a "dual regulatory" effect on germination, as higher concentrations of SW inhibit germination, while lower concentrations have a germination promoting effect (Light et al. 2002). 3,4,5-Trimethylfuran-2(5H)-one (2,3,4-trimethylbut-2-enolide), a compound isolated from plant-derived smoke, was found to be responsible for its germination inhibiting activity (Light et al. 2010). Therefore, in order to maximize its stimulant biological activity, the SW must be diluted with water before use, usually at ratios of 1:250, 1:500, 1:1000, 1:1500 and 1:2000 (v/v), depending on the plant species (Van Staden et al. 2004).

SW is a material that is cheap, economical and easy to use, used in very low concentrations and stored for many years. Different researchers have conducted various studies to obtain SW. Many researchers have tried to prepare SW by using different plant materials, burning plant materials at different temperatures and times, and attempted to establish the active application range. Knowing the KAR concentration in the SW is crucial for biological studies. There remains no standard method for obtaining SW and using concentration. Although this importance is known, an optimum, fast and cheap method has yet to be developed. Our research aims to develop a standard method for the preparation of SW (optimal burning time and temperature) and to find the most active range for germination to compare the SW obtained for the germination test with the commercially available KAR_1 substance statistically. In addition, the research aims to determine the KAR_1 concentration in the obtained SW with HPLC device and discuss it in relation to other studies in the literature. The most important point that distinguishes this study from other studies is that it is the first study in terms of developing a standard method for obtaining SW. In addition, since the amount of KAR_1 in the SW obtained by this method is higher than other studies in the literature, we believe that it will be a source literature for future studies.

2. Material and Methods

2.1. Material

In the research, the seeds of the Şalak apricot variety of *Prunus armeniaca* L., belonging to the Rosaceae family, were used as material (Figure 3). The seeds were obtained from Iğdır University Agricultural Application and Research Center (TUAM). After the fleshy parts of the apricot was separated and washed, it was dried in a cool and shaded place.

Figure 3- Tree, fruit and seed form of the material used, respectively

2.2. Methods

2.2.1. Smoke water preparation

SW was obtained by burning 1 kg of *Medicago sativa* L. straw in 1 L sterile distilled water in a Carbolite brand ELF 11/6B model laboratory oven, allowing the smoke to dissolve in the water in the erlen (Figure 4). The *Medicago sativa* L. straw was burned at 275 ºC for 60 minutes until it turned to ash. In order for the smoke to dissolve more in water, an ice pack was placed under the filtering flask. The obtained SW was stored at +4 ºC until used.

Figure 4- A) The process of burning the *Medicago sativa* L. straw in the laboratory oven, B) The smoke water obtained

2.2.2. Measurement of karrikin content in smoke water by HPLC

A HPLC analysis was carried out at Iğdır University Research Laboratory Practice and Research Center. The HPLC was performed with an Agilent 1260 Infinity Series device containing a Diode Array detector. A Zorbax C18 (4.6×250 mm) reverse phase column with a diameter of 5 micrometers and an injection volume of 20 micrometers was used as the column. The column was eluted with 50% acetonitrile at 1 mL/d 30 ºC for 10 minutes and then with 50% H_2O for 10 minutes. UV absorbance was measured at 325/4 nm wavelength. KAR_1 (Toronto Research Chemicals Canada) was then added to the HPLC device library. KAR_1 was introduced to the device at a concentration of 5.08076 ng μL^{-1}. Then, in order to measure the amount of KAR in the SW, the SW was introduced to the device; the retention time and the amount of KAR in SW given by the device were evaluated.

2.2.3. Sterilization of seed and other materials to be used in the study

The sterilization of seeds was carried out according to Kemeç Hürkan and Akı (2022). Before sterilization, 2 mg of KAR_1 was dissolved in 2 mL of chloroform solvent, and then diluted (0.01 µM, 0.1 µM, 1 µM, 5 µM, 10 µM) from the stock solution (1000 ppm) was used. SW was used by dilution from the stock solution (1:100, 1:500, 1:1000, 1:5000, 1:10000) obtained after burning the plant material. SW was passed through filter paper before being used in the study. SW and KAR_1 were sterilized by being passed through a membrane filter (0.22 µm) before use. Glass materials (petri dishes, magentas, measuring tape, flask, beaker, bottles, etc.), filter papers, forceps, scalpels and distilled water to be used in the study were sterilized in an autoclave at 121 °C under 1.2 atmospheres pressure for 15 minutes.

2.2.4. Germination of seeds

Seeds were sown under aseptic conditions, and a sterile cabinet with laminar flow and HEPA filter was used for this. After the testa part of the sterilized seeds was peeled, they were transferred to petri dishes with filter paper inside. Each group consisted of 50 seeds and seeds were sown in 10 replications, with 5 seeds per petri dish.

Then, according to the experimental groups, each of them was wetted with the previously prepared solutions (Figure 5).

The control group was only wetted with sterile distilled water. Petri dishes were wrapped with cling film to prevent the moist filter papers from drying out. The seeds were stored under dark conditions at 4 ºC±1 (wet stratification in cold) until germination. Seeds germinated after 1 week and germinated seeds were recorded for statistical data.

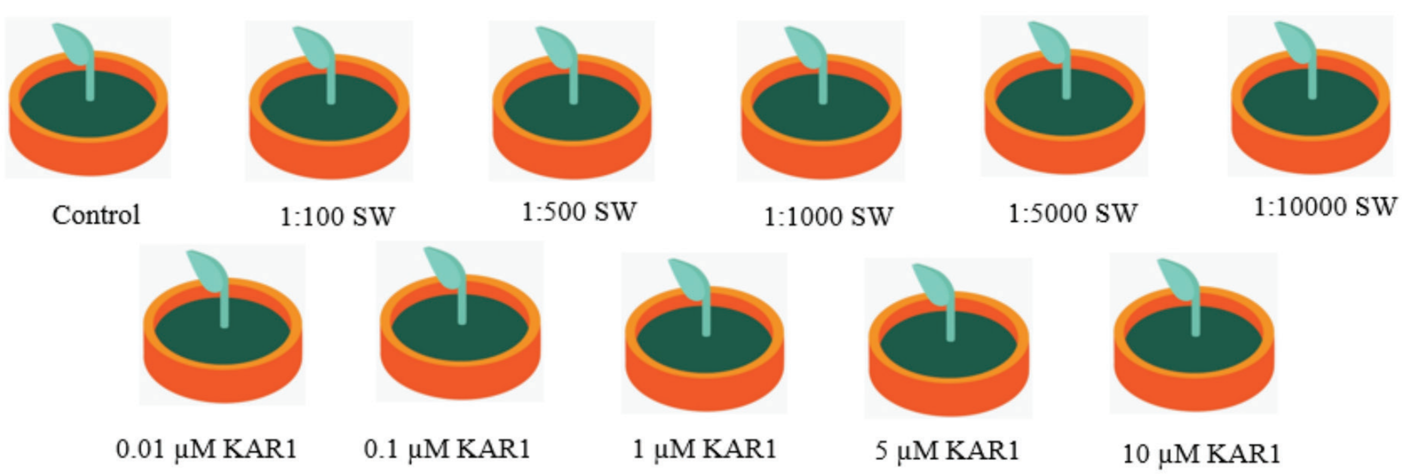

Figure 5- Experimental groups prepared for germination study
SW: Smoke water, KAR_1: $Karrikin_1$

2.2.5. Statistical analysis

All of the data obtained from this study were evaluated by making ANOVA in the XLSTAT 2021 statistical package program according to the randomized plots trial design. After the statistically significant transactions were determined, the differences between the averages were determined using the Duncan test at the p=0.05 level. The obtained data are given in tables as mean ± standard deviation.

3. Results and Discussion

3.1. Results

3.1.1. HPLC measurements

The KAR_1 substance added to the device library to measure the amount of KAR in the SW by HPLC gave a clear peak in 4.287 minutes (Figure 6A). Then, SW was introduced to the device to measure the amount of KAR_1 in the SW and it was determined that the KAR_1 concentration was calculated as 8.70398 ng/µl in 4.328 minutes (Figure 6B). Since SW consists of 71 compounds, unlike KAR_1, the HPLC peak was flactual.

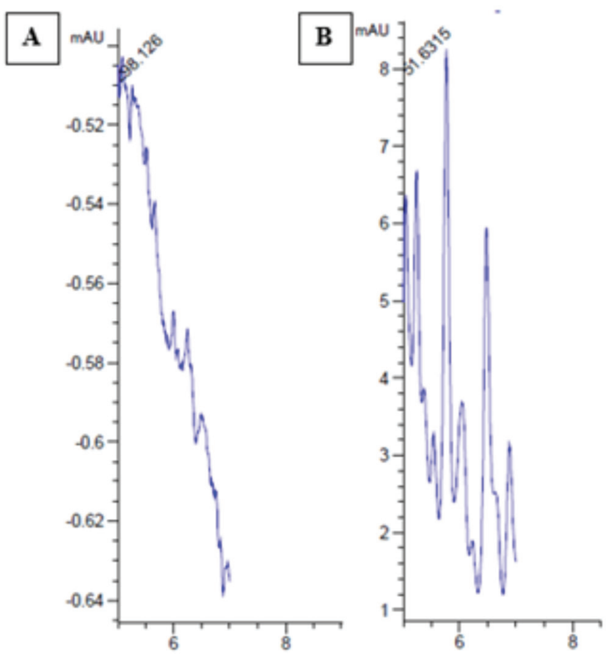

Figure 6- A) Retention time of KAR_1 substance, B) Retention time of smoke water

3.1.2. Seed germination percentage test

The seeds in the experimental groups started to germinate after 3 days. After one week, the germination rates were determined.

As a result of the statistical analysis, the difference between the groups in terms of germination was found to be significant ($p<0.05$). The highest rate of germination was 1:1000 SW (60%) and 1 µM KAR_1 (72%) (Table 1). It was observed that the germination increased as the concentration decreased in the SW substance at the concentrations tried throughout the study, and the germination increased as the concentration increased in the KAR_1 substance. For this purpose, for concentration optimization in germination, experimental groups were formed at 1:5000 and 1:1000 concentrations in the SW group and at 5 µM and 10 µM concentrations in the KAR_1 group, and germination rates were determined. According to the study, it was observed that germination decreased at the concentrations tested. In terms of germination, it was determined that the concentration of 1:1000 in the SW group and 1 µM in the KAR_1 group was appropriate.

Table 1- Effects of experimental groups on germination

Experimental groups	*Germinated seed (%)*
control	34.00±0.229^{ef}
1:100 SW	40.00±0.387^{cdef}
1:500 SW	50.00±0.403^{bcd}
1:1000 SW	60.00±0.387^{ab}
1:5000 SW	28.00±0.245^{fg}
1:10000 SW	30.00±0.512^{fg}
0,01 µM KAR_1	46.00±0.229^{bcde}
0,1 µM KAR_1	54.00±0.391^{bc}
1 µM KAR_1	72.00±0.332^{a}
5 µM KAR_1	38.00±0.350^{def}
10 µM KAR_1	18.00±0.350^{g}
p<0.002	

Values with different superscript letters in the same column are significantly different from each other (p<0.05; Duncan's test). SW: Smoke Water, KAR_1: $Karrikin_1$

4. Discussion

We think that the amount of KAR in the SW content may vary depending on the burned material, the burning temperature, the burning time and the amount of the burned material. In the majority of studies, forest floor vegetation such as *T. triandra* Forssk, *Heteropogon contortus* Beauv. ex Roemer & J. A. Schultes, *Tristachya leucothrix* Trin. ex Nees, *Hyparrhenia hirta* (L.) Staph, *Aristida junciformis* Trin. & Rupr, *Cymbopogon validus* Stapf ex Burtt Davy, *Cynodon dactylon* (L.) Pers., leaves and wood parts of plants in the form of shrubs and trees such as *Eucalyptus lanceolatus* Labill., *Eucalyptus camaldulensis* Dehnh., *Saraca asoca* (Roxb.) Willd., *Morus alba* L., *Ficus religiosa*. (L.) Forssk, *Passerina vulgaris* Meisn., straw, urban waste plant materials, paper, sugar cane, cellulose, glucose, xylose and glycine were used as the burning material (Flematti et al. 2009; Kochanek et al. 2016; Gupta et al. 2019; Hrdlička et al. 2019; Shabir et al. 2021). In our research, *Medicago sativa* L. straw was used as the burning material. Straw contains 30-40% cellulose and 20-30% xylose (Artık et al. 1993; Yüksel 2017). According to Flematti et al. (2004), synthetic cellulose, glucose, xylose and glycine were burned and the amount of KAR in their content was determined. They found KAR in xylose + glycine > xylose > cellulose > glucose, respectively (Flematti et al. 2011). According to the literature, it is thought that if the plant material is burned at 180-200 ºC for 30 minutes, it will be sufficient to release the substances that will promote germination (Flematti et al. 2015). The burning temperature and time typically used are between 180-200 ºC and 10-30 minutes (Brown & Van Staden 1997; Van Staden et al. 2004; Downes et al. 2013; Çatav et al. 2018a; Çatav et al. 2018b; Shabir et al. 2021), and in another study 450-730 ºC for 2-40 minutes (Kochanek et al. 2016). In most studies, the burning temperature and time were not specified, and the plant material is burned until ashes. In our research, the plant material was burned at 275 ºC for 60 minutes. We prepared a comprehensive table includes all the parameters and results which have been used to obtain SW in the literature (Table 2). According to comparison, we obtained the highest KAR_1 concentration in SW and believe that this may be due to the material we burned (*Medicago sativa* L. straw), the burning temperature, the burning time and the amount of the burned material. In addition, by placing an ice pack under the filtering flask where the smoke is dissolved, the faster and more effective dissolution of the gases in the water showed that more KAR_1 substance is held in the SW. In the literature, it is seen that the amount of KAR_1 obtained in the study (Gupta et al. 2019) in which plant material was burned at a rate of 1/1, like our study, is the closest result to our study. In other studies, even if proportionally more plant material was burned, the amount of KAR_1 obtained was found to be very low. This may be due to the difference in burning time and temperature. According to the study conducted by Kochanek et al. (2016), it is thought that the plant material should burn slowly, under low temperature and with large raw material quantities in order for the KAR_1 substance to be more concentrated in the SW. On the contrary, it is thought that if the plant material is burned quickly, under higher temperature or with a small amount of raw material, the KAR_1 substance is consumed more quickly and deteriorates, and it is not formed effectively. It is therefore estimated to be present only in low concentrations in SW mixtures produced under these conditions.

Table 2- Comparison of the literature and the data obtained in this study

		The amount of KAR_1 in the smoke water	***[1]Present study: Literature ratio***	***Temperature (ºC)***	***Duration (min.)***	***Burned material***	***Amount of material burned***
The data of this research		8.70398 ng µL^{-1}		275	60	Medicago sativa	1 kg/1 L
	Gupta et al. (2019)	1.71148±2300 ng µL^{-1} (2018 data)	5:1	The plant material was burn up until ashes Fynbos leaves *Passerina vulgaris*, *Themeda triandra*		*Themeda triandra*, Heteropogon *contortus*, *Tristachya leucothrix*, *Hyparrhenia hirta*, *Aristida junciformis*, *Cymbopogon validus*	26 kg/26 L
		0.00123±3.2 ng µL^{-1} (1993 data)	7076:1			5 kg/500 mL	
		0.00488±1.4 ng µL^{-1} (1998 data)	1784:1				
References	Hrdlička et al. (2019)	0.00176±4.3 ng µL^{-1}	4945:1	-	45	Fynbos leaves	
		0.00488±1.4 ng µL^{-1}	1784:1			*Passerina vulgaris*, *Themeda triandra*	5 kg/500 mL
		0.00949±1.5 ng µL^{-1}	917:1			*Themeda triandra*	
		0.00141±0.4 ng µL^{-1}	6173:1			*Themeda triandra*	10 kg/500 mL
		0.01117±3.7 ng µL^{-1}	779:1			Fynbos leaves	5 kg/500 mL
		0.01525±7.9 ng µL^{-1}	571:1	The plant material was burn up until ashes		Commercial smoke water (brand and model not specified)	
	Kochanek et al. (2016)	0.069±8.8 ng µL^{-1}	126:1	590	28-29	Pyrolytic liquid (wood vinegar)	250 kg/20 L

[1]KAR_1 ratios obtained in this study according to the literature

At high concentrations (1:100 or less dilution) SW inhibits germination. However, lower concentrations (1:1000 dilution) significantly increase germination compared to control (Light et al. 2002). Consistent with the literature data, in our study, it was observed that for apricot seeds, germination increased as SW concentration decreased, and germination increased as KAR_1 concentration increased. The best germination optimization for SW was obtained at a concentration of 1:1000, and for KAR_1 at a concentration of 1mM. A decrease in the germination percentage was observed at 1:5000 and 1:10000 SW concentrations. We think that this is because as the dilution rate increases, the density of the KAR_1 substance decreases as well as the 3,4,5-Trimethylfuran-2(5H)-one substance present in its content, and it slows down the germination rate. A decrease in germination percentage was also observed at 5 mM and 10 nM KAR_1 concentrations. We think that the reason for this is that the increased concentration creates a toxic effect for the seed and thus slows the germination rate.

5. Conclusions

With the SW preparation method designed in this study, the results show that low cost, simple and very high concentrations of SW can be obtained. Unlike other systems, adjusting the burning temperature and time allows for more control and a more effective performance. In this way, much more plant growth regulators will be produced in quantity than other plant growth regulators that can be stored and used for many years. Commercially available plant growth regulators (GA, auxin, cytokinin, ABA, strigolactone, etc.) are both very expensive, not stored for long periods, and are also sensitive to heat. SW has the potential to be used in many fields of plant sciences such as agriculture, horticulture and laboratories. SW is an economic substance that supports seed germination, shoot, root and plant growth even at very low concentrations. We think that this study will help other researchers working in this field in terms of SW preparation method and optimization. SW can potentially be used in plant tissue culture, molecular biology, plant physiology, agriculture, and plant protection. In addition, farmers may see the benefits of it in agriculture if the SW generating apparatus is made for large-scale commercial use.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: Y.K.H., C.A., Design: Y.K.H., C.A., Data Collection or Processing: Y.K.H., Analysis or Interpretation: Y.K.H., C.A., Literature Search: Y.K.H., Writing: Y.K.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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