



**EFFECT OF SMOKE (LEAVES OF *EUCALYPTUS* SP. AND *AZADIRACHTA* SP.)
TREATED WATER ON TOMATO SEED GERMINATION**

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Abstract: *Accidentally or incidentally forests catch fire and the smoke is released from the burning vegetation which contains chemical constituents like butenolide. This smoke gets dissolved in the rainwater and influences the germination of various seeds. The effect of smoke treated water has been investigated in case of horticultural crops throughout the world. In the present investigation, the smoke is collected from burning Eucalyptus sp. and Azadirachta sp. leaves and dissolved in the water. The smoke water treated seeds were tested for their germination percentage and seedling vigor by using moist Petri plate method. The smoke treated water has exhibited an inhibitory effect on tomato seeds resulted in the low percentage of germination and seedling vigor. The study of effect of smoke treated water for protein expression in the seeds has been carried out with SDS PAGE. The molecular weight of the inhibitory proteins responsible inhibition has been identified by using a molecular marker (Bovine serum albumin).The result reveals the presence of two inhibitory proteins with K Da values of 42.6 and 47.125.*

Keywords: *Smoke, Seeds, germination, seedling vigor, Identification of inhibitory Proteins, SDS PAGE.*

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INTRODUCTION

Seed Germination is a process by which a seed embryo develops into a seedling and subsequently a plant. It involves the reactivation of the emergence of the radicle or seed root and plumule or shoot. There are several conditions or factors affecting the seed germination like embryo must be alive i.e., seed must be viable, any dormancy requirements that prevent germination must be overcome, proper environmental conditions must exist for germination. Some seeds require specialized “pre-conditioning” before they will germinate. The seed germination includes three phases of the germination according to physiological process namely activation, digestion and translocation, seedling growth. There are several environmental factors that affect the process of germination. These are considered to be secondary dormancy factors. They are water, light, oxygen, temperature. Seed dormancy refers in failure of a viable seed to germinate even in the presence of favorable environmental conditions. During this dormancy period, growth, development and physical activity are temporarily suspended. This minimizes the metabolic activity and therefore helps an organism to conserve energy.

Different methods are adapted for breaking the seed dormancy which includes scarification, charate, mulching, use of hot water, dry heat, acids, chemicals, germination temperatures, fire etc., The tomato nurseries are practicing temperature method to break the dormancy, in which the seeded trays are incubated in a closed facility to increase the temperature. This takes four to five days to germinate the seeds. The present investigation considered the use of smoke treated water from the two different sources i.e. *Azadirachta* sp., *Eucalyptus* sp. Leaves to check the possibility to break the dormancy of tomato seeds. The identification of responsible proteins has also been taken for the study, using SDS PAGE to give overall conclusion of their germination i.e, the percentage of germination and seedling vigor.

Seed dormancy provides a mechanism for plants to delay germination until conditions are optimal for survival of the next generation. Dormancy release is regulated by a combination of environmental and endogenous signals with both synergistic and competing effects. Molecular studies of dormancy have correlated changes in transcriptomes, proteomes, and hormone levels with dormancy states ranging from deep primary or secondary dormancy to varying degrees of release. The balance of abscisic acid (ABA): gibberellin (GA) levels and sensitivity is a major, but not the sole, regulator of dormancy status. ABA promotes



dormancy induction and maintenance, whereas GA promotes progression from release through germination; environmental signals regulate this balance by modifying the expression of biosynthetic and catabolic enzymes. Mediators of environmental and hormonal response include both positive and negative regulators, many of which are feedback-regulated to enhance or attenuate the response. The net result is a slightly heterogeneous response, thereby providing more temporal options for successful germination [1]. Smoke can be applied to seeds immediately before sowing, or the seeds may be pretreated and stored until conditions are appropriate for sowing. Both smoke and aqueous smoke-water is active in this respect. The active constituent is volatile, thermo stable, water soluble and long-lasting in aqueous solution and in the soil. Attempts to identify the active compound and to determine the mechanism of action have been unsuccessful. It is becoming increasingly clear that smoke as a germination (or growth regulating) cue must have evolved as part, or as a consequence of fire, as an evolutionary factor. As such, it is probably a very old development and serves as an additional protection mechanism to ensure germination at optimal times for seedling survival [2]. In the 1990s, however, it became apparent that one of the most important inducers of germination in post-fire environments is smoke itself. This discovery raised a number of questions. For example, what is the factor in smoke that induces germination? Is there more than one factor? Will a given species respond differently to smoke from different sources? How does smoke interact with other dormancy-breaking cues, and what is the physiological mechanism by which smoke acts? The answers to these questions are by no means clear researchers are still wrestling with phenomenological descriptions of this process [3]. The slow combustion of dry or green plant material from many sources produces compounds that are water-soluble and that stimulate the germination of many seeds. The active principals are apparently produced around 160°C to 200°C and are volatilized at higher temperatures. How widespread a phenomenon is smoke-induced germination? Surveys, most of which have focused on species from fire-prone areas, have revealed that more species respond favorably to smoke than do not. The positive effect of smoke on seed germination, however, is by no means limited to species native to fire-prone habitats. In many species, the effects of smoke are astounding. Smoke, for example, has been reported to enhance the germination of the South African plants *Erica clavisepala* and *Restio*



festuciformis by more than 7,000% and 25,000%, respectively. There can be complex, species-specific interactions between smoke and other environmental factors. In some cases, smoke is a better enhancer of germination than heat, or heat and smoke act synergistically to enhance germination. Factors such as seed age, light levels, temperature, and hydration levels can also influence the extent of smoke-induced germination. Whatever its mechanism, smoke's ability in enabling seeds to rapidly overcome dormancy is long lasting. Seeds treated with smoke retain an enhanced ability to germinate even after 1 year of storage [4]. The observed cuticular changes are consistent with the hypothesis that volatiles in smoke exert a surfactant-like reaction to break seed dormancy in the California chaparral annual *Emmenanthe penduliflora*. [5]. The site of action of smoke water in seed was investigated and found to reside in part in the seed coat in *S. affine*, and the embryo and/or endosperm in *A. leucocephalus*. The smoke chemical(s) overcame multiple dormancy mechanisms in *S. affine* and *A. leucocephalus* whereas gibberellic acid (GA) and zeatin were unable to break dormancy. Mechanism of dormancy relief by smoke water was not the same as GA and zeatin. These data indicate that there are good prospects using imbibition with smoke water as a pre-treatment for seeds in the horticulture and land restoration activities [6].

A butenolide, isolated from smoke, can overcome the detrimental effects of extreme temperatures during tomato seed germination:

The butenolide, 3-methyl-2H-furo [2, 3-c] pyran-2-one, is a highly active compound isolated from plant-derived smoke. This compound is known to stimulate seed germination in a wide range of plants akin to smoke or aqueous extracts of smoke. The present study attempted to elucidate the role of the butenolide in overcoming detrimental effects of low and high temperatures on tomato seed germination and seedling growth. The germination percentage followed a parabolic curve for temperatures ranging from 10 to 40°C, with 25°C being the optimum for all treatments. Control seeds showed radicle emergence at two extreme temperatures (10 and 40°C) and seedlings failed to develop further, even upon prolonged incubation. By comparison the butenolide-treated seeds grew into phenotypically normal seedlings at these non-optimum temperatures. The smoke–water-treated seeds had an intermediate response as only a fraction of germinated seed developed into normal seedlings. Seedling vigor indices as well as seedling weight were significantly higher



($p \leq 0.05$) for butenolide-treated seeds at all temperatures. Furthermore, seedlings developed in the presence of the butenolide had about a 1:1 correspondence between root and shoot length. Butenolide-treated seeds grew better than the control seeds in the temperature shift experiments. A gradual decline in the vigour index values was recorded with an increased duration of incubation at the extreme temperatures. Results of the present study are very important from a horticultural point of view as they indicate the potential use of the butenolide compound in restoring normal seed germination and seedling establishment in tomato below and above optimum temperatures. [7].

METHODOLOGY

Sources of Smoke Treated water & Tomato Seeds: Smoke water has been collected from burning dry leaves of *Azadirachta* sp. and *Eucalyptus* sp. in the laboratory. The leaves were collected in Madanapalle Institute of Technology and Science campus, Madanapalle, Chittoor (D), Andhra Pradesh, INDIA. Tomato seeds of Vigro brand, Golden seeds of variety F1 Vaishnavi (2082), LOT-8111004, Germination percentage of 70% and purity 98% were used in investigation.

Seed Germination Test: Seed germination tests measure the number of healthy well developed seedlings under laboratory conditions. The standard laboratory tests were applied for 200 seeds. Seeds were germinated under optimum environment conditions for an optimum period of time according to the species in the moist Petri plates. Distinctions are made between normal, abnormal and the dead seed. Based on physical assessment damaged seeds were removed.

The percentage of germination was calculated as follows.

$$\text{Percentage of seed Germination} = \frac{\text{Total number of germinated seeds}}{\text{Total number of healthy seeds}} \times 100$$

Seedling Vigor Test: Seedling vigor is the measure of the quality of the seed and involves the viability of the seed, the germination percentage, germination rate and the strength of the seedlings produced. Hence seedling vigor test considered as the closest measure for potential field performance of seed. Seedling vigor was calculated as follows:

$$\text{Seedling vigor} = (\text{Shoot length} + \text{Root length}) \times \text{Germination percentage}$$



Separation of Proteins: The SDS-Poly Acrylamide Gel Electrophoresis technique has been used to study the extracted proteins from germinated seeds, in which any charged ion or molecule moves when placed in an electric field depends on its net charge, size, shape and the applied current.

This can be represented by the following equation

$$V = E \times q / f$$

V - Velocity of movement of the molecule E - Electric field in volts/cm

q - Net electrical charge f - The frictional coefficient of the molecule

Around 100 tomato seedlings were crushed in 3.5ml sample buffer, transferred into an eppendorf tube and were kept in water bath at 100C for 3-5mins. The heated samples were centrifuged at 12000g for 10min. The supernatant was collected in fresh eppendorf tube and was stored in a refrigerator.

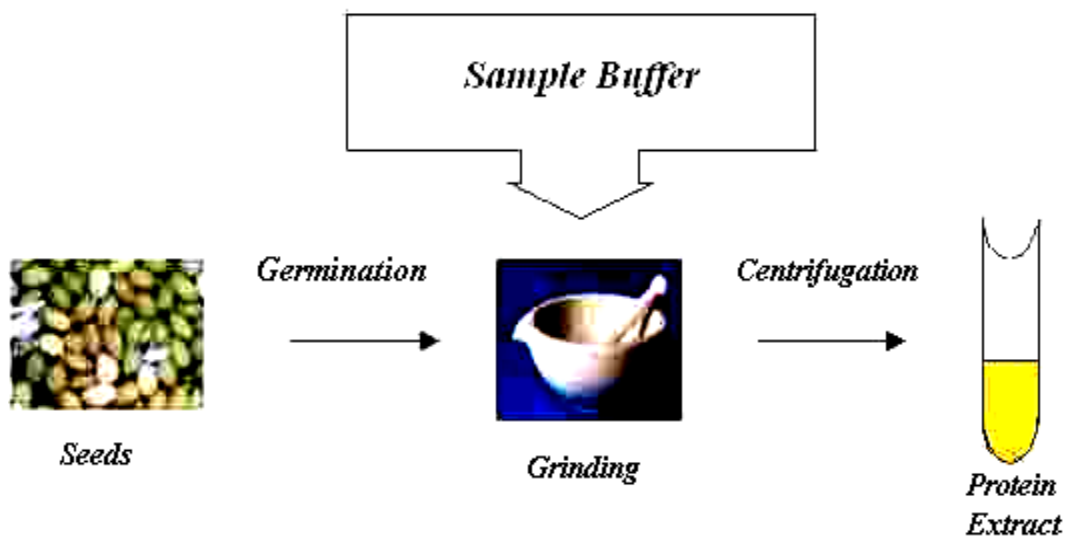


Figure No.1: Procedure for Sample Preparation

20 μ l of each of the samples were loaded in a predetermined order at the bottom of the wells (15% separating gel & 5% stacking gel) with the help of micro syringe. It was attached to an electric power supply. The positive electrode was connected to the bottom of the reservoir. A voltage of 50v/cm was applied to the gel and the gel was run till the bromophenol dye front reaches 0.5cm from the lower edge of the gel. The glass plate was removed from the electrophoresis apparatus and the orientation of the gel was marked by cutting a corner from the bottom of the gel that is closest to the left most well. The gel was



immersed in 5 volumes of staining solution and was left undisturbed for at least 4 hours at room temperature.

The gel was de stained by soaking the gel in the de staining solution and left undisturbed for 4 - 8 hours. Change the solution 3 or 4 times. Then the destained gel was collected and by observing the distances moved by protein bands and the tracking dye, the R_m values of the seed proteins were estimated using the formula,

$$R_m = \frac{\text{Distance between origin and band}}{\text{Distance between origin and the tracking dye}}$$

RESULTS AND DISCUSSION:

The percentages of germination in case of distilled water treated seeds (control) was 89.5%, *Azadirachta* sp. leaves' smoke water treated seeds was 72% and *Eucalyptus* sp. leaves' smoke water treated water was 74%. The percentage of germination was maximum in case of distilled water treated seeds and least in the case of *Azadirachta* sp. leaves' smoke water treated seeds (Table No.1) has been observed.

Table No.1: The comparison between Average root length, Average shoot length, Germination percentage & Seedling Vigor among distilled water, leaves of *Azadirachta* sp. & *Eucalyptus* sp. smoke water treated seeds.

Type of Water	Avg., Root Length	Avg., Shoot Length	% of Germination	Seedling Vigor
DW	1.12	0.80	89.5 %	171.84
NSW	0.43	0.36	72.0 %	56.88
ESW	0.60	0.37	74.0 %	74.64

DW – Distilled water treated seeds; NSW – *Azadirachta* sp. leaves smoke water treated Seeds; ESW – *Eucalyptus* sp. leaves smoke water treated seeds

The seedling vigor of distilled water treated seeds was 171.84, *Azadirachta* sp. leaves' smoke water treated seeds was 56.8 and *Eucalyptus* sp. leaves' smoke water treated seeds was 74.64. The seedling vigor is maximum in distilled water treated seeds whereas it was minimum in *Azadirachta* sp. leaves smoke water treated seeds (Table No: 1).

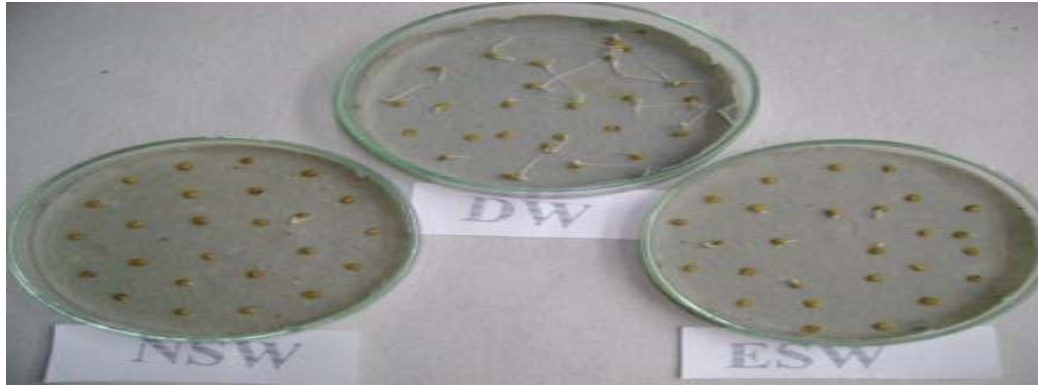


Figure No. 2: Moist Petriplates showing germination percentage and seedling vigor tests for distilled water (DW) treated seeds, *Azadirachta* sp. leaves' smoke water (NSW) treated seeds and *Eucalyptus* sp. leaves' smoke water (ESW) treated seeds.

The seeds in three petriplates clearly show the difference in the germination percentage and seedling vigor of three i.e., Distilled, *Azadirachta* sp. leaves' smoke and *Eucalyptus* sp. leaves' smoke water treated seeds. The result shows that *Azadirachta* sp. leaves smoke water treated seeds shows the least percentage of germination and seedling vigor compared to the other two. Distilled water treated seeds shows higher percentage of germination and seedling vigor than other two (Figure No.2).

Identification of Proteins:

The phenotypes were finally influenced by their genes expression. The gene expression generally occurs at different levels of the growth stages, also in seedling level. The protein level expression has been studied by using the method, SDS PAGE, where the protein banding patterns were observed to estimate the molecular weights.

Based on the distances moved by protein bands and the tracking dye, Rm values of the germinated seed proteins were estimated. Electrophoretic migration of a protein is inversely proportional to the molecular weight of the protein.

$$R_m \propto 1/\text{Mol wt.}$$

Such that the molecular weight of unknown protein is calculated as follows:

$$\text{Mol wt. of unknown protein} = \frac{\text{Mol wt. of marker} \times R_m \text{ value of marker}}{R_m \text{ value of unknown protein}}$$

$$R_m = \frac{\text{Distance between origin and band}}{\text{Distance between origin and the tracking dye}}$$

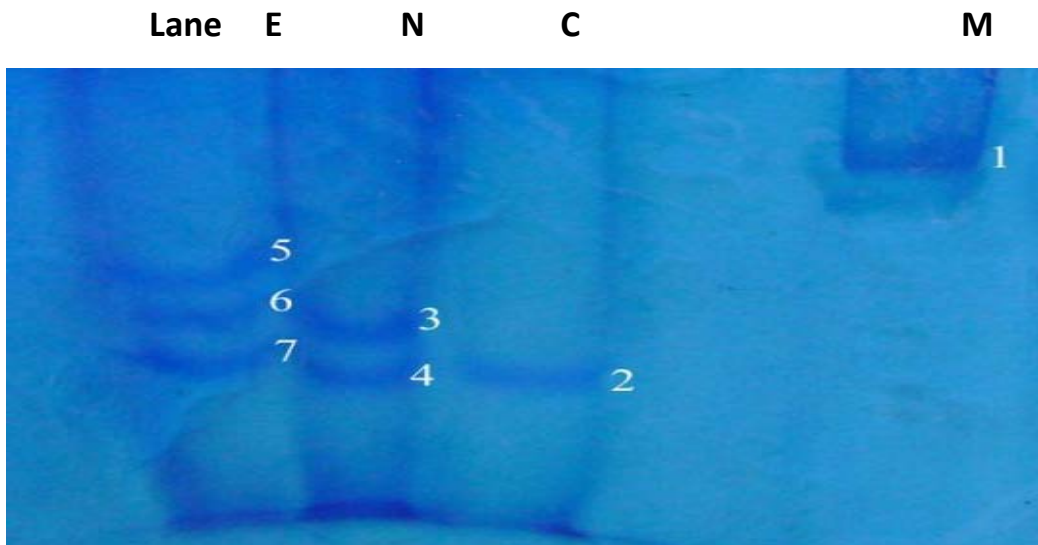


Figure No. 3: SDS PAGE Result showing comparison of germinated seed proteins. Lane M- Marker(Bovine Serum Albumin of Molecular Weight 66 KDa); C- Control (Distilled water treated); N- Azadirachta sp. leaves smoke water treated; E- Eucalyptus sp. leaves smoke water treated.

In the SDS PAGE result (Figure No. 3), it was observed that there is one common band in all the three lanes i.e., *Eucalyptus sp.*, *Azadirachta sp.* and control. In lane N one extra band and in case of lane E two extra bands were observed. This result indicates the presence of inhibitory proteins, one in *Azadirachta sp.* and two in *Eucalyptus sp.* among respective leaves smoke water treated germinated seeds.

Table No. 2: R_m values of marker, other protein bands, calculated molecular weights, comparison of SDS PAGE Result with seedling vigor.

S.No	Protein Band	R _m Value	Molecular weight (K.Da)	Seedling vigor
1.	Marker (BSA)(band 1)	0.377	66.00	--
2.	Lane C (band 2)	0.622	40.00	171.84
3.	Lane N (band 3)	0.584	42.60	56.88
4.	Lane N (band 4)	0.622	40.00	
5.	Lane E (band 5)	0.528	47.12	74.64
6.	Lane E (band 6)	0.584	42.60	
7.	Lane E (band 7)	0.622	40.00	



The molecular weights of unknown inhibitory proteins have been obtained from Rm values and marker molecular weight. The inhibitory proteins were **42.6KDa** & **47.125KDa** and compared with seedling vigor (Table No.2).

CONCLUSION:

In the present investigation, two different aqueous smoke solutions and distilled water were used for phenotypes and protein expression studies. The percentage of seed germination, seedling vigor test and SDS PAGE techniques were used. The both smoke treated water results have shown low seed germination percentage and seedling vigor in comparison with the distilled water treated seeds. The obtained result reveals that the aqueous smoke solutions treated germinated seeds have expressed the inhibitory proteins. The results were correlated with phenotypes.

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