

## Research Article

## The changes of 2,4 Dinitrophenol substance applied to corn seeds in AOX and ATP synthase gene expression against chilling stress

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### ABSTRACT

Plants get stressed when they are out of optimum living conditions. When the stress conditions exceed toleration level, they cause lots of sequential damages that are very difficult to repair for physical, biochemical and molecular mechanism of plants. The recent studies intended for strengthening resistance mechanism at gene level is also one of the popular research subjects. In this context, clearing up genes supplying resistance for plants (especially as food) which are exposed to instant temperature changes may provide convenience for other researchers to prevent loss of yield. In this study, changes of genes belonging to the enzyme named alternative oxidase (AOX) which is known to be active at low temperatures and located in mitochondrial ETS (electron transport system) by applying a substance named 2,4 dinitrophenol on corn seeds (*Zea mays*) were tried to be determined exogenously. It is known that AOX transfers electrons to oxygen to prevent occurring reactive oxygen species with accelerating respiration at low temperatures. Besides, it is also known as that AOX causes available energy spread as heat since it prevents occurrence of necessary electrochemical gradient for ATP synthesis. It was seen that dinitrophenol (DNP) known as slimming medicine in literature reviews is a chemical substance which disrupts electrochemical gradient and inhibits ATP synthesis, spreads available energy as heat. This similarity between AOX and DNP has directed us to research working mechanism of DNP and AOX. Finally, it was seen that DNP increases resistance against cold by stimulating AOX gene expression and repressing ATP synthase expression.

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### 1. Introduction

Low temperature occurred below 10°C is a factor which restricts to grow up plants and causes loss of yield (Yang et al., 2005). It brings about a lot of damage on variety of plants morphologically, physically and molecularly (Creencia and Bramlage, 1971; Mahajan and Tuteja, 2005; Kim et al., 2011; Duan et al., 2012). Before the low temperature makes morphologic changes, it appears by the corruption happening in physical mechanism. One of the most important reasons of these corruptions is reactive oxygen species (ROS) consisting of metabolic defects. Superoxide, hydrogenperoxide and hydroxyl radicals start up the reactions of cellular damage and they cause lipid peroxidation, protein oxidation and also DNA and RNA damages. Even these damages cause plant death (Heath and Packer, 1968; Asada, 1997; Mittler, 2002; Apel and Hirt, 2004). One of the mechanism low temperature affects negatively and is the first location of ROS produced is photosynthesis and the other one is respiration mechanism (Eaks, 1960; Watada and Morris, 1966). One of the most important effects of low temperature is the situation that is blocked ATP synthesis by accelerating respiration during electron transfer to water in oxidative phosphorylation (Lieberman et al., 1958; Lewis and Workman, 1964). This event is called as uncoupling. Although this situation seems

negatively, indeed it is necessary for the protection of respiration mechanism. As known, one of the essential purposes of respiration is electron transfer to oxygen and water formation, the other one is production of ATP which is the power supply of alive metabolism. It was observed that electrons are transferred to oxygen in the plants exposed to cold stress but ATP synthesis decreases, additionally it was also observed that the plants tolerate cold damage. When these events were researched in detail, it was observed that the energy stemming from available proton (H<sup>+</sup>) gradient cannot come out as ATP synthesis and the plants heat themselves internally with spreading this energy as heat. (Borecky and Vercesi, 2005; Wang et al., 2011). It can be understood in literature review, this mechanism is called as Alternative Respiratory Way (Vanlerberghe and McIntosh, 1997; McIntosh et al., 1998; Zhou and Solomos, 1998; Calegario et al., 2003). The essential enzyme in this way is Alternative Oxidase (AOX). AOX has been an enzyme on which was studied since it was explored and there are informative studies both as enzyme activity and molecularly. (Calegario et al., 2003; Vanlerberghe, 2013; O'Leary and Plaxton, 2016; Saha et al., 2016). But the question is that are there any substances or enzymes helping AOX or working as it in Alternative Respiratory Way?

This question reminded us uncoupling protonofores. Uncoupling protonofores which are able to dissolve both in

water and in lipid are substances deforming electrochemical gradient being pumped to the hole between mitochondrial membranes during electron transfer in oxidative phosphorylation and essential for ATP synthesis by remanding protons to matrix. That is, protonofores make inner membrane permeable for protons and they block occurrence of free energy ( $\Delta\mu H$ ) related to sufficient large chemical proton gradient so as to continue ATP synthesis. (Vercesi et al., 1995; Volkov and Mwesigwa, 2001; Jezek et al., 2001; Kowaltowski et al., 2001; Kadenbach, 2003). Protonofores can penetrate to the lipid phase of a membrane and they can carry one proton throughout the membrane. They can separate from a membrane potential or proton concentration. Therefore some energy occurs but it is not adequate for ATP synthesis (O'Brien et al., 1978) and this available energy spreads as heat. That is, it can be said that protonofores have almost the same mission as AOX. The difference is that AOX cannot pump protons, protonofores cannot carry electrons. FCCP (trifluoro methoxy phenyl hydrazone or carbonyl cyanide), 2,4 DNP (2,4 Dinitrophenol), oligomycin, momensin, CCCP (carbonyl cyanide m-chlorophenyl hydrazone) might be examples of these substances. These substances are used in low concentration for health area and medical sector. However, 2,4 DNP was chosen from these substances, which has never been tried in plants, was used on animals and humans in advance, low cost, even has the effect of antioxidant at low concentration.

2,4 DNP was first defined by Noomis and Lipman in 1948 and it has been known as uncoupling of phosphorylation during electron transfer since then. 2,4 DNP has been accepted as a classical uncoupling oxidative phosphorylation since that time (Racker, 1965). It was also used as a slimming medicine because of its heating feature in 1930's.

Because of all these, corn (*Zea mays*) which was exposed to cold stress, has high economic value and is a summer plant was used in this study. By applying exogenously low concentration of 2,4 DNP substance to the plant, it is tried to be determined both whether it increases the resistance against cold molecularly and physiologically and the relation of alternative respiratory way. This study is original in literature.

## 2. Materials and methods

### 2.1. Growing plants and making implementations

Corn plant (*Zea mays*) which has financial value, is delicate to cold and is sowed in Turkey was used in the study. Seeds were supplied by May seed which is registered by the name of Hido and the experiment was made with the help of this type of seed. Before the seeds were planted, they were washed with alcohol rated 96% quickly for a while and they were surface-sterilized in sodium hypochlorite rated 5% for 5 minutes. Afterwards, they were washed with a lot of pure water and they were left to puff for 5 hours. At the end of the

period, they were planted in hydroponic culture and they were left for growing at room temperature (25 °C and moisture 65%, 14-10 light/night period). At the ninth day of growing seeds, 4 mM SHAM, DNP with 75  $\mu M$  concentrations applications were made by spraying on leaves. Then, except the ones in control group, plants were harvested by keeping in cold (5-9 °C day/night) for 96 hours. Thus, application groups were created as Control, Cold, Cold + 4 mM SHAM, Cold + 4 mM SHAM + 75  $\mu M$  DNP, Cold + 75  $\mu M$  DNP and 75  $\mu M$  DNP respectively.

### 2.2. Determination of lipid peroxidation degree

Lipid peroxidation was measured by estimating malondialdehyde (MDA), a product of lipid peroxidation, using a thiobarbituric acid reaction Velikova et al. (2000). The changes of absorbance were recorded at 532 nm, and the values corresponding to nonspecific absorption (600 nm) were subtracted. The content of MDA (ng g<sup>-1</sup> FW) was calculated by using the molar extinction coefficient 155 (mM cm<sup>-1</sup>).

### 2.3. Determination of reactive oxygen species

Similar sized (for thickness) rootstocks were selected for the method expressed by Elstner and Heupel (1976) was used to quantify superoxide production. To calculate the production rate of superoxide, sodium nitrite was used as a standard solution.

The hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content was assayed as described by Velikova et al. (2000). The content of H<sub>2</sub>O<sub>2</sub> was calculated by comparison with a standard calibration curve.

### 2.4. RNA extraction and real-time PCR

Total RNA was isolated from 100 mg leaf samples by using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol in QiaCube apparatus. The concentration of purified total RNA samples was defined using Qiaxpert apparatus. Prior to cDNA synthesis, agarose gel electrophoresis was used for determination of the total RNA quality and integrity. cDNA was synthesized from 2 ng of total RNA using NanoScript 2 RT Kit (Primer Design) according to the manufacturer's protocol. Gene specific primers were purchased from Qiagen. The sequences of the used primers were given in Table 1. The quantitative real-time PCR reactions included 5ml cDNA solutions, 10ml Master Mix (2xqPCR Master Mix), 1ml of each primer, 4ml distilled water in 20ml final volume. The amplification reactions were performed in a Thermal Cycler (Qiagen, Rotor Gene Q) with initial denaturing temperature of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, annealing temperatures of each primer 15 s and the extension at 58 °C for 1 min. Analysis of the relative gene expression was done based on Qiagen Data Analysis Center. The specificity of the amplifications was checked based on the melting curves resulting from heating the amplicons from 50 to 95 °C (Erdal and Turk, 2016).

**Table 1.** Amplification primers for RT-PCR.

Genes	Sequence	Start Position	Strand	Length	Primer Tm	Scale
AOX2	TTGCTTTAGCCATGGGAGA	410	F	20	55.97	0.05 umol
	CTCATGGTGTCTGGTGAGGT	454	R	20	56.04	0.05 umol
ATP	ATTGCCAAACCGTGCTACAG	1043	F	20	56.21	0.05 umol
SYNTASE	GCTCAGCAGGAAGGATAGGT	1182	R	20	55.89	0.05 umol

### 2.5. Statistical analysis

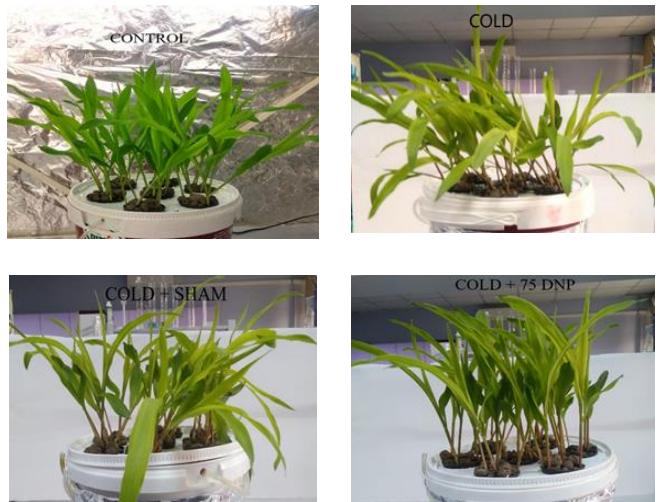
Total RNA was isolated from 100 mg leaf samples by using the data are the average of six replicates per treatment. It was analyzed by ANOVA with the Duncan's multiple range test with SPSS 25.0 to separate means, and the result indicated in tables such a way that alphabets a, b, c, d, e and f represents first, second, third, fourth, fifth and sixth levels of statistical significance, respectively. The difference between the means indicated by the same letter in the same column is not statistically significant in the  $P < 0.05$ . All analysis is considered as significant at  $P \leq 0.05$ .

### 3. Results and Discussion

Respiration mechanism is quite complex and consists of a lot of intertwined reactions. A possible hitch in a small part of this mechanism shows itself with some corruptions in related reactions too. During mitochondrial respiration, especially the stage of producing ATP (oxidative phosphorylation) smoothly is very important. In case of any hitch, electron leaks will trigger to occur ROS because electron transfer takes place in this stage. That's why, natural toleration mechanisms are available in plant body against risk factors. Alternative Respiratory Way (AOX) is also one of these mechanisms. While AOX has already been studied, the discovery of the elements triggering more effective usage of this mechanism means that the plant is less damaged of stress conditions, particularly cold stress. Common point of 2,4 DNP with Alternative Respiratory Way is that they are capable of eliminating ROS and internal heating the plant (Skulachev, 1998; Jin et al., 2004; Genisel, 2012). Thus, while the plant is able to protect itself against cold and also it prevents electron leaks from harming mechanism.

In this study, it has been tried to be determined the effect of exogenous 2,4 DNP substance on alternative respiratory way in corn seeds in cold conditions. In this context, at the beginning, it was determined that cellular disruption rate and superoxide content which is premise of ROS that causes this disruption, then whether stress in plant occurs against cold by identifying the amount of  $H_2O_2$ . The amount of MDA was also determined so as to be able to see what size these identifications damage to the cells. After being sure about stress occurrence, it was examined how 2,4 DNP influences to AOX and ATP synthase at gene level. Before the harvest, the plants were surely observed for their physical changes (fig 1). It was seen that the leaves of control plant are greener and more upright than the ones of cold group. It was observed that the leaves of the plants 75  $\mu$ M DNP applied are more upright and livelier than the ones of cold and cold-SHAM group. It was tried to determine whether 2,4 DNP affects to AOX by inhibiting AOX metabolism in SHAM

(salicylic hydroxamic acid) group. These changes have made us think that 2,4 DNP has different morphological effect from other groups.



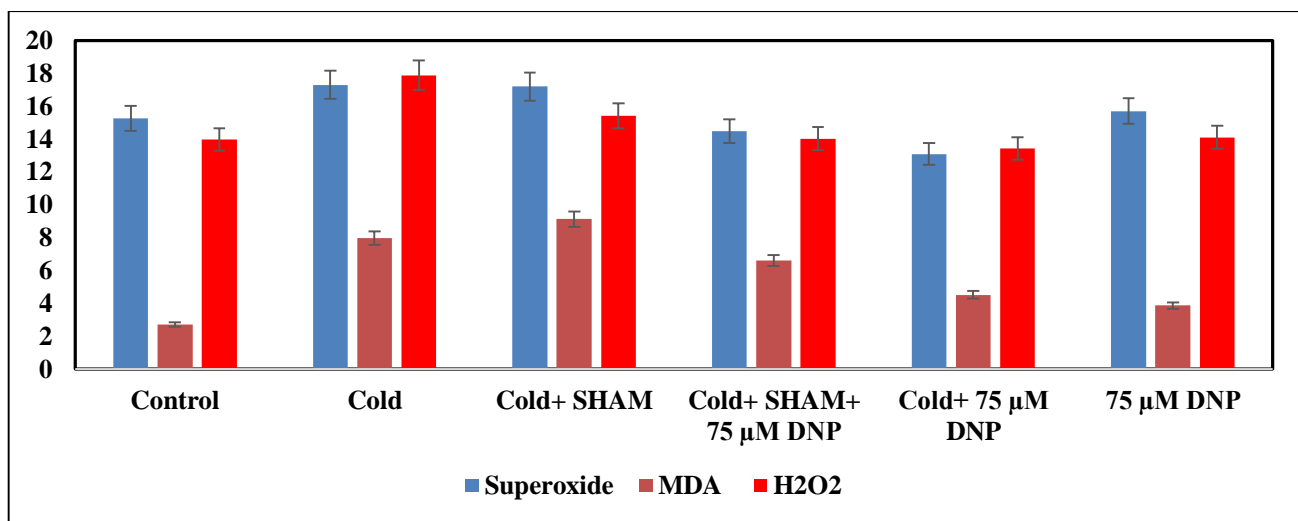
**Figure 1.** The effect of 2,4 Dinitrophenol on corn plant against cold

It was reported that the production point of much of ROS is mitochondria and chloroplasts in the plants. Especially, ROS production occurs in the plants at low temperatures (De Santis et al., 1999; Kratsch and Wise, 2000; De Virville et al., 2002). Electrons are transported in internal membranes of mitochondria. It has been noticed that the respiration is speeded up when the plant gets stressed. (Erdal and Genisel, 2016). Speeding up the respiration means sufficient electrons are not be able to convey to oxygen and exposed. Exposed electrons cause ROS production. (Alscher et al., 1997). The premise converting ROSs each other (such as hydroxyl radical, peroxides, hydroxy fatty acids) is an oxidant substance called superoxide ( $O_2^-$ ). It has been reported that rising superoxide in cells may cause cellular disruption and increasing lipid peroxidation (LPO) rate (Mutlu, 2009). Additionally, superoxide composite turns into  $H_2O_2$  (hydrogen peroxide) by activating antioxidant defense system in order to eliminate harmful outputs of oxidant substances. This compound is also an oxidant but it is less harmful than superoxide. It is an intermediate product consisting during electron transfer to water. It is turned into water by antioxidant substances and enzymes. Determining  $O_2^-$  and  $H_2O_2$  compounds in cells in large amount and being lipid peroxidation (LPO) rate high mean that a lot of metabolic glitches will happen. For this reason, the stress level has been determined by seeing  $O_2^-$  and  $H_2O_2$  contents

and LPO rate in corn seeds exposed to cold stress. Because it is molecular study, only stress level has been identified by these parameters and determined whether stress is or not and also it has been observed the effects of these parameters on target genes. Antioxidant defense system is the subject of another study.

Before gene parameters, we must look out the results of these three parameters; these parameters have shown us that cellular disruption rate rises visibly in corn seeds exposed cold stress by increasing superoxide and hydrogen peroxide (Table 2 and Figure 2). These data have been results expected in literature. On the other hand, this rate shows itself with the results equivalent to control in the groups with 75  $\mu\text{M}$  DNP application and this situation shows that corn seeds are hardly affected by the cold. Even, it is possible to

see the positive effect of DNP substance on the groups with SHAM substance which is toxic with the aim of inhibiting the gene of AOX. This situation takes us to two results based on the literature: the first one is that DNP substance inhibits ATP synthesis and thus it ensures that available energy releases as heat (Gifford, 1967; Volkov et al., 1998). Thus, the plant may not have been affected by cold heating itself internally. The second one is that the amount of DNP with antioxidant effect has decreased the amount of superoxide and hydrogen peroxide and so the plant may have been protected from cold damage significantly (Korde et al., 2005). Of course, each probability may have come true together. In order to get more certain information about these features of DNP, it is necessary that all antioxidant defense system must be studied in detail.



**Figure 2.** The changes 2,4 DNP made in superoxide, hydrogenperoxide and MDA content of corn plant exposed to cold stress

**Table 2.** The changes 2,4 DNP made in superoxide, hydrogen peroxide and MDA content of corn plant exposed to cold stress

Groups	Superoxide content (μg g <sup>-1</sup> )	MDA content (LPO) (nmol g <sup>-1</sup> )	H <sub>2</sub> O <sub>2</sub> Content(μg g <sup>-1</sup> )
Control	15.27 ± 0.072c*	2.73 ± 0.014a	13.98 ± 0.091b
Cold	17.3 ± 0.044e	7.98 ± 0.073e	17.88 ± 0.028e
Cold+ SHAM	17.2 ± 0.061e	9.13 ± 0.090f	15.42 ± 0.056d
Cold+ SHAM+ 75 μM DNP	14.5 ± 0.077b	6.62 ± 0.025e	14.02 ± 0.051b
Cold+ 75 μM DNP	13.1 ± 0.036a	4.52 ± 0.012c	13.44 ± 0.019a
75 μM DNP	15.7 ± 0.081d	3.87 ± 0.062b	14.1 ± 0.060c

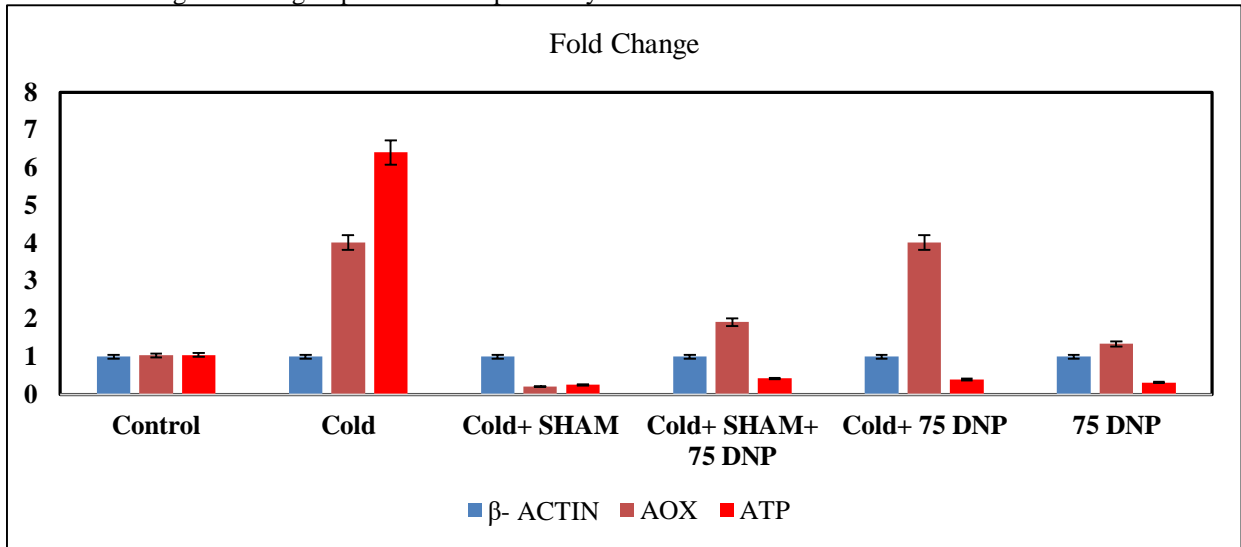
\*: The difference between the means indicated by the same letter in the same column is not statistically significant in the  $p < 0.05$ .

So as to determine complete effect of 2,4 DNP substance on AOX and ATP synthase, the expressions of AOX and ATP synthase at gene level have been examined in another stage of the study. In literature, it was concluded that the expression of AOX gene rises and the expression of ATP synthase gene decreases in cold conditions. (Li et al., 2013; Erdal and Genisel, 2016; Krebler et al., 2019). The obtained data were in accordance with literature researches. On the other hand, any effect of 2,4 DNP substance on AOX has not

been reported. However, it was reported that the energy releases as heat on account of the fact that 2,4 DNP disrupts proton gradient and the available energy is not enough for ATP synthesis (Goldgof et al., 2014). This situation means that 2,4 DNP decreases ATP synthesis. Current data were consistent with this finding (Table 3, Figure 3 and 4).

While 2,4 DNP increased the resistance against cold stress, it decreased ATP synthesis. Besides, we can say that 2,4 DNP increases the expression of AOX in corn plant

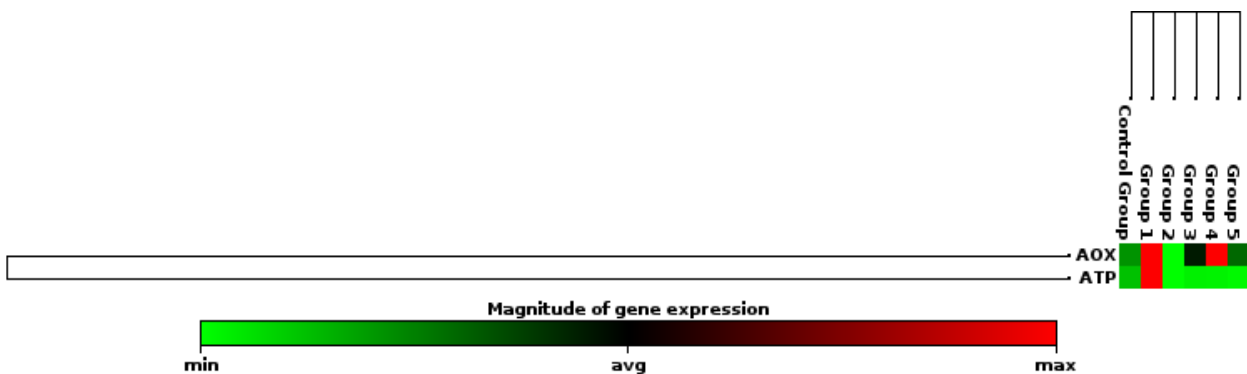
exposed to cold stress and nonetheless, it has affected the expression of AOX gene in the group with SHAM positively.



**Figure 3.** Fold change graphic showing increasing and decreasing expressions of alternative oxidase and ATP syntase gene according to the applications groups of corn plant exposed to cold stress

**Table 3.** CT values showing increasing and decreasing expressions of alternative oxidase and ATPsyntase gene according to the applications groups of corn plant exposed to cold stress

Groups	B-ACTIN	AOX	ATP Syntase
Control	17.06	17.11	16.36
Cold	31.47	29.51	28.08
Cold+ SHAM	17.82	20.15	19.10
Cold+ SHAM+ 75 $\mu$ M DNP	16.83	15.94	17.37
Cold+ 75 $\mu$ M DNP	31.47	29.51	32.13
75 $\mu$ M DNP	18.14	17.76	19.10



**Figure 4.** Clustergram map showing increasing and decreasing expressions of alternative oxidase and ATPsyntase gene according to the applications groups of corn plant exposed to cold stress (Group1: Cold, Group 2: Cold+ SHAM, Group 3: Cold+ SHAM+ 75 DNP, Group 4: Cold+ 75 DNP, Group 5: 75 DNP).

#### 4. Conclusion

Consequently, 2,4 DNP substance of which experiment was not made on plants exposed to cold stress affected corn seeds positively against cold stress. It was able to make this positive effect by both increasing internal heat and helping activation other internal heating mechanism. When the study started, it was aimed to determine the effect of 2,4 DNP on AOX gene.

However, when the data gathered, 2,4 DNP substance not only increased internal heat but also was able to tolerate the stress significantly. This observation was a silver lining for further phases of this study. We wish this study helps the scientists who research photosynthesis, respiratory mechanisms and stress.

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## Authors' Contributions

Z. Kilic carried out experiments, analyzed data, prepared graphs and wrote draft of this article. R. Dumlupınar provided planting materials, experimental fields and helped in conducting experiments in laboratory. Also, he finalized the draft of this article.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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