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ORIGINAL ARTICLES

Gene Expression of Heat Stress on Protein and Antioxidant Enzyme Activities of Two *Lupinus* Species

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ABSTRACT

Two *Lupinus* species *albus* and *luteus* (white and yellow lupins, respectively) were studied for heat stress tolerance. Plants were grown under different time and temperature regimes (30, 35, 40, 42°C/30 min and 42°C for 1, 2, 3 and 4 h, respectively) compared with the constant degree of 25°C as a control in growth chambers. The experiments were conducted twice with three replicates. The variations in heat stress-induced protein and antioxidant enzyme activities among both species were investigated under heat stress tolerant (HST), synthesis of many proteins is repressed and some of them, which are called heat-shock proteins (HSPs), start to be synthesized from 12.5 to 37.7 KDa and above in *L. albus*, and 27.24 to 61.56 KDa in *L. luteus*. The most of protein bands were disappeared at 42˚C/4 h in *L. luteus* compared with the control. Electrophoretic patterns of the peroxidase (POX) isozyme tested showed four bands under the control and three bands under different temperatures treatments in both species. However, polyphenol oxidase (PPO) isozyme appeared three bands for *L. albus* with different intensity, while the band (No.3) was disappeared in the control and all different temperatures treatments of *L. luteus*. Finally biochemical parameters as alterations in the leave storage protein banding patterns and change in the expression level of peroxidase and polyphenol oxidase isozymes were differed in the different temperatures treatments in both species.

Key words: Heat shock protein (HSP), tolerant genotypes, peroxidases (POX), polyphenyl oxidase (PPO), SDS-PAGE.

Introduction

Lupinus sp. are an important plant of leguminous crops for both animal and human nutrition because of their high seed protein and oil content (Pinheiro *et al*., 2004 and 2005). The study of changes in gene activation, transcription, translation and expression induced by high temperature may help to understand the ability of *Lupinus* sp. plants to tolerate changes in environmental conditions that occur during the growth of plant. Heat is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to plant growth and development. Heat stress, due to high ambient temperatures, is a serious of threat to crop production worldwide (Hall, 2001). In nature, plants are often subjected to environmental fluctuations; among those are salt, drought and heat stresses. In response to, both biotic and abiotic stresses, plants synthesize a wide set of proteins including chitinases and peroxidases and up-regulated phenylalanine ammonialyase, (PAL) (De jaegher *et al*., 1985). In fact, plants submitted to environmental aggressions develop their defensive reactions involving an activation of many genes as a response to various stimulations (Collinge and Slusarenko, 1987; Sturm and Chrispeels, 1990). Many of the reported studies on heat-shock protein structure and function were investigated through in *vitro* cultured cells (Nover and Scharf, 1984). Heat stress tolerant (HST) is one of the most important constraints on crop production that adversely affects the vegetative and reproductive processes of plant and ultimately reduces yielding and quality (Abdul-Baki 1991; Gruda 2005). Plants respond to HST by changing their metabolic pathways to acclimatize to high temperature. Under HST, synthesis of many proteins is repressed and some of them, which are called heat shock proteins (HSPs), start to be synthesized (Vierling 1991). HSPs synthesis is induced by a rapid rise in temperature of approximately l0ºC or more above the optimal growth temperature (Nover and Scharf, 1997). The authors reported that HSPs plays a major role in mitigating the deleterious effects of heat-induced protein denaturation. Moreover, physiological responses of plants to HST such as the damage of structure and the disorder of physiological metabolism were documented (Vierling, 1991). Although the damage and death of cells are caused by extreme HST, many plants can survive in otherwise lethal high-temperature regimes if they are first subjected to a pretreatment at non-lethal high temperature (Vierling, l99l). Exposure of plants to elevated temperatures for short term, heat shock HST results in a complex set of gene expressions, selective translation of mRNA-encodings HSP, thereby

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enhancing thermotolerance and improving cellular survival to subsequent HST (Nover *et al.*, 1989; Gong *et al.*, 2001).

High temperature is a major factor which significantly affects plant productivity in arid and semi arid regions (Bray *et al.*, 2000). Exposure of plants to abiotic stresses results in production of reactive oxygen species (ROSs) as by products, which damage the cellular components (Noctor and Foyer, 1998). Plants have developed a series of enzymatic and non-enzymatic detoxification systems to counteract ROS, and protect cells from oxidative damage (Sairam and Tyagi, 2004). The Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and glutathione reductase (GR) function in detoxification of superoxide and H_2O_2 (Mittler, 2002).

High temperature adversely affected various physiological and metabolic processes (Wahid *et al*., 2007). Peroxidases are ubiquitous class of plant enzymes that oxidize a variety of hydrogen donors at the expense of hydrogen peroxide (McEldoon *et al.*, 1995). Protective mechanisms that could scavenge the peroxidatively produced free radicals and peroxides have evolved within the seed, keeping this deleterious compound to a minimum (Bowler *et al* ., 1992).

Since the *Lupinus* is winter crop and characterized by high protein content. So, it is important to study the impact of high temperature on protein and isozymes electrophoresis at the early stages of germination. Therefore, the variation in heat stress-induced antioxidant enzyme activities and protein among *L. albus* and *L. luteus* must be studied.

Objective of this study was to investigate the effect of heat stress on the protein banding patterns of *Lupinus* species using SDS-PAGE and the antioxidant isozymes peroxidas (POX) and polyphenol oxidases (PPO).

Materials and Methods

Plant Materials:

Two accessions of *L. albus* CGN 10107 and *L. luteus* CGN 10125 (origin country: Netherlands) supported from (Centre for Genetic Resources, The Netherlands).

Heat shock experiment:

Seeds of two species from *Lupinus* (*L. albus* and *L. luteus*) were soaked in distilled water for 48 h. The germinated seedlings were then transferred to sterile test tubes (one per tube) containing 15 cm^3 of distilled water, filter paper and nutrient solution (Ferreira and Davies, 1987). The seedlings were kept on sterile filter paper for two weeks inside the test tubes. The nutrient solution was replaced every day by adding fresh solution into the test tubes in a laminar flow hood. Heat shock treatment was given to 14 days old seedlings by gradual increase in temperature from 25 to 42˚C over 4 h in a water bath. Afterwards, the plants were divided into three sets; the first set was exposed to different temperature degrees for constant time period 30, 35, 40 and 42˚C/30 min. The second set was exposed to constant temperature degree for different time periods 42˚C for 1, 2, 3 and 4 h. The third set had the plants which did not receive the heat shock treatment (25˚C) (control). At the end of the experiment, the plant were harvested and storage at – 80 ºC until further use methods.

SDS-protein electrophoresis:

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the protein banding patterns of the two species from *Lupinus* under the control and heat shock treatments. Protein fractionation was performed according to the method of Laemmli (1970) as modified by Studier (1973).

Isozymes electrophoresis:

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted according to Stegemann *et al.*, (1985) to identify isozyme variations between control and heat shock treatments on the two *lupinus* sp. Two isozymes systems; peroxidase and polyphenol oxidase were analyzed. After electrophoresis, gels were stained according to their enzyme systems with the appropriate substrate and chemical solutions and then incubated at room temperature in dark for complete staining. For peroxidase, benzidine-dihydrochloride HCl of 0.125 gm and 2 ml glacial acetic acid and was completed with distilled water up to 50 ml. Gel was placed into this solution and 5 drops of hydrogen peroxide was added. The gel was incubated at room temperature until bands appear (Brown, 1978). For polyphenol oxidase, 100 ml of sodium phosphate buffer 0.1 M at pH 6.8, 15 mg cathecol and 50 mg sulfanilic acid were used. The Gel was placed into this solution and incubated at 30°C for 30 min until bands appeared.

Results and Discussion

1. SDS-protein electrophoresis:

The electrophoretic banding patterns of proteins extracted from the leaves of *L. albus* under control and different heat stresses are shown in (Fig. 1 and Table 1). Fifteen bands ranging from 12.5 to 100 bp; 12 bands were polymorphic with 80% polymorphism varied between heat treatments compared with the control. The other three bands were commonly numbers at molecular weights (MWs) of 91.5, 77 and 37.7 KDa, respectively. Band number 1 at MW of 100 KDa appeared in the control, 30˚C/30 min, 42˚C/30 min, and 42˚C/3 h treatments, respectively. The visible changes in SDS-PAGE patterns were the appearance of few new bands like bands number 4,5,7,9,11,12,13 and 14 at MWs of 68.8, 61.3, 47.6, 39.1, 19.40, 18.3, 15.1 and 14.4 KDa, respectively, appeared under 42˚C/30 min, 42˚C/1 h, 42˚C/2 hrs, 42˚C/3 h, and 42˚C/4 h treatments respectively, while disappeared from control these bands may be due to heat stress and presence of heat shock protein. The highly visible changes were found in band number 10 at MW of 37.7 KDa which showed oscillating of density and intensity. This increased density and intensity may be due to heat stresses. Band number 15 at MW of 12.5 KDa appeared in the control and all treatments except under 35˚C/30 min with some intensity changes and no obvious trend, this band could be considered as a negative specific marker for heat stress shock. The presence of some bands in the treatments and their absence from the control could be referring to activation of some genes related to heat stress. These results were in an agreement with (Yamaguchi *et al*., 1995) who noted that, there was gradual increase in the number of new protein bands with the increase in heat-shock temperature and the maximum protein band was observed when the heat-shock treatment of 40˚C was given for 2 h. These new protein bands may be the HSPs. Abdel-Tawab and Allam, (2002) confirmed that newly synthesized bands indicated that salt stress induced related gene(s) to produce these salt inducible proteins. Compatible with these results, Cherian and Ferreira, (2010) reported that under heat stress (HST), synthesis of many proteins is repressed and some of them, which are called heat shock proteins (HSPs), start to be synthesized from 15 to 30 KDa. Also Arora *et al*., (2000) professed that water stress induced the accumulation of stress responsive proteins belonging to dehydrin group (25-60 KDa) and/or aquaporins (25-30 KDa).

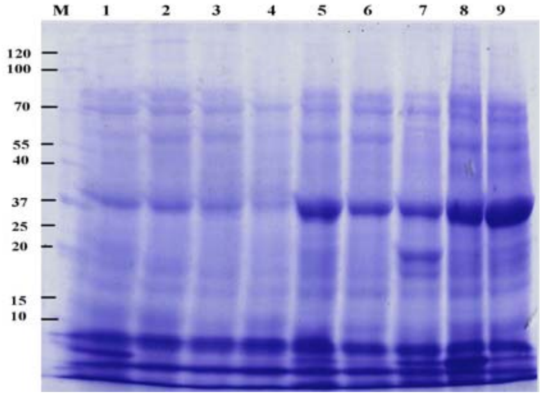

Fig. 1: SDS-PAGE of water soluble protein fraction for *L. albus* under the control and different temperatures treatments. Lane 1=25°C (control), lane 2= 30°C/30 min, lane 3= 35°C/30 min, lane 4= 40°C/30 min, lane 5= 42°C/30 min and lanes 6, 7, 8 and 9 = 42°C for1, 2, 3 and 4 hrs, respectively.

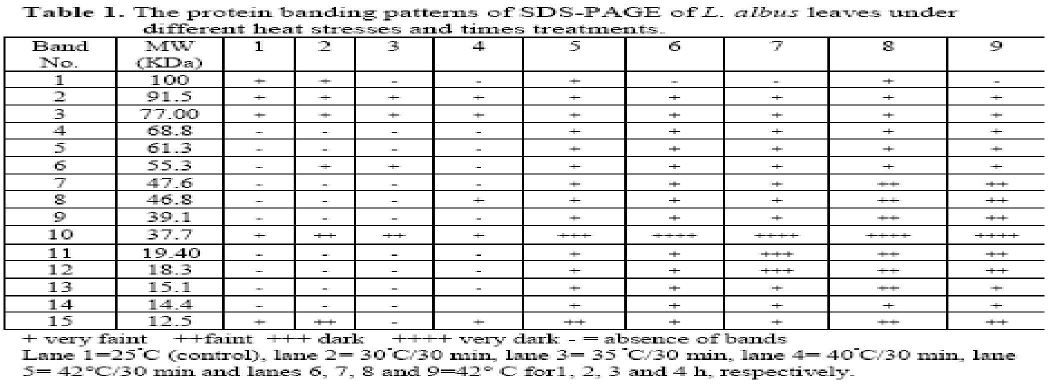

Table 1. The protein banding patterns of SDS-PAGE of *L. albus* leaves under different heat stresses and times treatments.

Band No.	MW (KDa)	1	2	3	4	5	6	7	8	9
1	100	+	+	-	-	+	-	-	+	-
2	91.5	+	+	+	+	+	+	+	+	+
3	77.00	+	+	+	+	+	+	+	+	+
4	68.8	-	-	-	-	+	+	+	+	+
5	61.3	-	-	-	-	+	+	+	+	+
6	55.3	-	+	+	-	+	+	+	+	+
7	47.6	-	-	-	-	+	+	+	++	++
8	46.8	-	-	-	+	+	+	+	++	++
9	39.1	-	-	-	-	+	+	+	++	++
10	37.7	+	++	++	+	+++	++++	++++	++++	++++
11	19.40	-	-	-	-	+	+	+++	++	++
12	18.3	-	-	-	-	+	+	+++	++	++
13	15.1	-	-	-	-	+	+	+	++	+
14	14.4	-	-	-		+	+	+	+	+
15	12.5	+	++	-	+	++	+	+	++	++

\+ very faint ++faint +++ dark ++++ very dark - = absence of bands
Lane 1=25˚C (control), lane 2= 30˚C/30 min, lane 3= 35 ˚C/30 min, lane 4= 40˚C/30 min, lane 5= 42°C/30 min and lanes 6, 7, 8 and 9=42° C for1, 2, 3 and 4 h, respectively.

For *L. luteus* (Fig. 2 and Table 2), appeared a total number of 16 bands with different MWs ranged from 12.80 to 114.72 KDa. 12 bands were polymorphic with 75% polymorphism. The remaining four with different intensities were monomorphic bands with MWs (45.83, 36.03, 34.17 and 19.20 KDa). Bands number 1, 2, 3, 4, 7, 12, 13 and 14 with MWs of 114.72, 105.31, 98, 96.46, 57.17, 28.33, 27.24 and 26.86 KDa, respectively were detected in the control and all treatments except for 42˚C/4 h with different intensities, therefore they could be considered as a negative specific marker for heat stress shock. Band number 5 with MW of 69 appeared in all treatments except 40˚C/30 min and 42˚C/4 h. Bands number 6 and 8 with MWs 61.56 and 56.53, respectively revealed very dark in 42˚C/2 h due to heat stress shock. Band number 16 at MW 12.80 appeared with the control, 30˚C/30 min and 42˚C/4 h, respectively, while absent with all treatments. In case of *L. luteus* only few protein bands were observed with different changes intensities of bands observed with gradual increase in heat shock temperature. At 42˚C/4 h no new protein bands were observed in *L. luteus* which may be denaturation of the new protein band expressed earlier. These results confirmed that there was consistent difference in protein banding pattern between the studied *lupinus* treatments compared with the control. This modification in gene expression is due to the few conservative genes found in plants, which respond to high temperature. These results agree are with (Yang *et al.*, 2006) who found that upon exposure to high temperature, pattern of gene expression is changed, which is pivotal for thermo-tolerance. El-Fadly *et al.*, (2007) noted that there was an increase in number and intensity of some bands in wheat treated which might be attributed to water stresses. AL-Whaibi (2010) indicated that the first reaction to stress on the level of transcription in the plant included a group of stress response genes. These genes might have a crucial role in the response to different stresses, as well as the main role of systemic signals generated by the tissue exposed to stress. Cherian and Ferreira (2010) reported that under HST, synthesis of many proteins is repressed and some of them, which are called heat shock proteins (HSPs), start to be synthesized from 36 to 45 KDa and above. These results indicated that the modifications in the electrophoretic profiles of leaves proteins confirmed the ability of these heat stress treatments to alter gene expression in exposed plants.

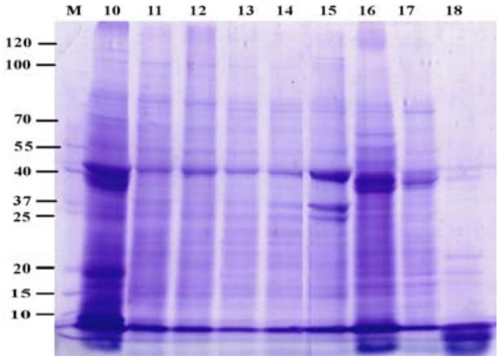

Fig. 2: SDS-PAGE of water soluble protein fraction for *L. leteus* under control and different temperatures treatments. Lane 1=25°C (control), lane 2= 30°C/30 min, lane 3= 35°C/30 min, lane 4= 40°C/30 min, lane 5= 42°C/30 min and lanes 6, 7, 8 and 9 = 42°C for 1, 2, 3 and 4 hrs, respectively.

Table 2. The protein banding patterns of SDS-PAGE of *L. luteus* leaves under different heat stresses and times treatments.

Band No.	MW (KDa)	1	2	3	4	5	6	7	8	9
1	114.72	++	+	+	+	+	++	+	+	-
2	105.31	++	++	++	++	+	+	+	+	-
3	98	++	++	++	+	++	++	++	+	-
4	96.46	+	+	+	++	++	++	++	++	-
5	69	++	+	+	-	+	+	+	+	-
6	61.56	++	-	+	+	+	+	+++	-	-
7	57.17	++	++	+	+	+	+++	+++	++	-
8	56.53	++	-	-	-	+	+	+++	-	-
9	45.83	++++	++	++	++	++	+++	++++	++	+
10	36.03	++++	+	+	+	+	+	++++	+++	+
11	34.17	++	+	+	+	+	+	+++	++	+
12	28.33	++	++	+	++	++	++++	+	+	-
13	27.24	+++	+	+	++	++	++++	++	+	-
14	26.86	+++	+	+	+	+	+	++	+	-
15	19.20	+++	+	+	+	+	+	++	+	+
16	12.80	+++	+	-	-	-	-	-	-	+

+ very faint ++faint +++ dark ++++ very dark - = absence of bands
Lane 1=25˚C (control), lane 2= 30˚C/30 min, lane 3= 35˚C/30 min, lane 4= 40 ˚C/30 min, lane 5= 42°C/30 min and lanes 6, 7, 8 and 9= 42°C for 1, 2, 3 and 4 h, respectively.

2. Isozymes electrophoresis:

2.1 Peroxidase (POX) isozymes:

The electrophoretic patterns of peroxidase enzymes of the treatments differed gently from that of control (Fig. 3 and Table 3). For *L. albus*, a total number of four bands were exhibited*;* band number 1 appeared in the control and 30˚C/30 min, 42˚C/2 h and 42˚C/3 h, respectively. Band number 2 appeared in all treatments with different intensities except the control and 30˚C/30 min. Band number 3 appeared in all treatments with different intensities except the control, 30˚C/30 min, 40˚C/30 min and 42˚C for 1 and 4 h, while band number 4 appeared in the control only and disappeared in all treatments which may be due to heat shock stress.

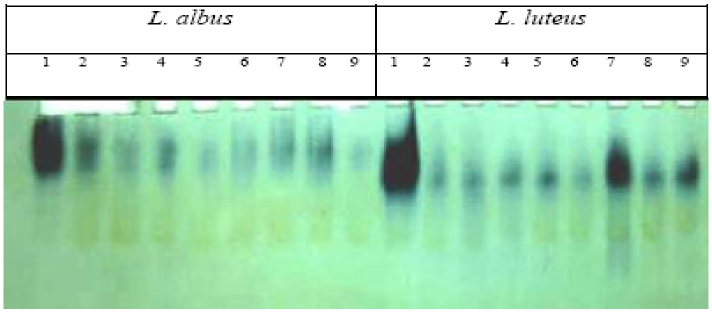

Fig. 3: Electrophoretic patterns in *L. albus and L. luteus* for proxidase isozymes under control and different temperatures treatments. Lane 1=25 °C (control), lane 2= 30°C /30 min, lane 3= 35°C/30 min, lane 4= 40°C/30 min, lane 5= 42°C/30 min and lanes 6, 7, 8 and 9 = 42°C for1, 2, 3 and 4 h, respectively.

Table 3. Effects of different heat stresses and times on peroxidase and polyphenol oxidas isozymes of leaves of *L. albus and L. luteus.*

Band No.	*L. albus*									*L. luteus*								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
Peroxidase isozymes																		
1	++++	++	-	-	-	-	+	+	-	++	-	-	-	-	-	++	-	-
2	-	-	++	+++	+	+	++	+++	+	++++	+	++	++	++	+	++++	+++	+++
3	-	-	++	-	++	-	+	+	-	-	+	+	+	+	+	++++	++	++
4	+++	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-
Total	**2**	**1**	**2**	**1**	**2**	**1**	**3**	**3**	**1**	**3**	**2**	**2**	**2**	**2**	**2**	**3**	**2**	**2**
Polyphenol oxidase isozymes																		
1	++++	+++	++	+++	-	++	++	++	-	+++	-	-	-	-	-	++	-	-
2	++++	+++	++	+++	++	+++	++++	++++	+	++++	++	++	+	+++	+	+++	+	++
3	++	++	+++	+++	++	+++	++	++	+	-	-	-	-	-	-	-	-	-
Total	**3**	**3**	**3**	**3**	**2**	**3**	**3**	**3**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**
Total	**5**	**4**	**5**	**4**	**4**	**4**	**6**	**6**	**3**	**5**	**3**	**3**	**3**	**3**	**3**	**5**	**3**	**3**

+ very faint ++faint +++ dark ++++ very dark - = absence of bands
Lane 1=25˚C (control), lane 2= 30˚C/30 min, lane 3= 35˚C/30 min, lane 4= 40˚C/30 min, lane 5= 42°C/30 min and lanes 6, 7, 8 and 9=42° C for 1, 2, 3 and 4 h, respectively.

For *L. luteus*, a total number of four bands were exhibited, band number 1 appeared only under the control and 42˚C/2 h, these may be due to denaturation of isozyme with heat stress. Band number 2 was monomorphic were detected in the control and all treatments with different intensities, band number 3 disappeared in the control and revealed in all treatments with different increase in intensity under high temperature 42 for 2, 3 and 4 h. Contrary, band number 4 appeared in the control only, while disappeared under all treatments. The preview results indicated that few bands disappeared or newly appeared in the heat stress shock as compared to control. These results resemble those obtained by Grouch *et al*., (1983) and Roy and Mandal, (2005) who reported that the reason for the decrease in peroxidase activity after roasting may be due to the protein denaturation. Rashed *et al*., (1994) found occurrence of different response in the decrease of intensity rather than in the isoforms of peroxidase in favor of salt tolerant genotype under stress. Pastori and Trippi (1992) reported that drought resistant maize showed greater induction of ascorbate peroxidase (APX) activity than sensitive plants. A variety behave as a tolerant only when it has sufficient compatibility to express different HSPs, as well as antioxidant enzymes in response to different abiotic stresses and proved the favorable condition to this protein in order to have their maximum activity to neutralize the free radicals which are produced because of oxidative burst. The HSPs present at particular stages also helps in protecting the important key enzymes of metaholic pathways from denaturation in response to heat stress. Also, El-Baz *et al*., (2003) used peroxidase isozyme and protein pattern as a marker for salt stress tolerance in cucumber plants, and found that, the profile of isoperoxidase was modified during salt stress conditions, also a new subset of proteins induced by salt stress compared to control plant was observed. This behavior may be due to its ability to tolerate salt stress or due to the effect of salt stress which may cause some shift in gene expression.

2.2 Polyphenol oxidase (PPO) isozymes:

Three bands appeared for polyphenol oxidase in *L. albus* (Fig. 4 and Table 3). The band number 1 appeared in the control and all treatments except 42˚C/30 min and 42˚C/4 h. Bands number 2 and 3 were commonly detected in the control and all treatments with different densities and intensities. It is noted that, the variations occurred in a different treatments when compared with the control confirmed that treatments trigger the induction of compounds that regulate the induction or the activity of the defense compounds of *L. albus*.

Two bands appeared for polyphenol oxidase in *L. luteus* (Fig. 4 and Table 3). Band number 1 was revealed only in the control and 42˚C/2 h. Band number 2 appeared in the control and all treatments with different densities and intensities. The PPO-activity decreased with increasing temperature and time and showed very little activity under 42˚C for 1 and 3 h. This may be due to PPO activity was lost upon different heat stress and time and then enzyme activity. These results were in an agreement with El-Beltagi *et al.,* (2010) who found that the reason for the decrease in polyphenol oxidase activity after roasting may be due to protein denaturation. Also, Gautam *et al*., (1998) and Montavon and Bortlik, (2004) reported that roasting treatments decline polyphenol oxidase activity in mushroom and coffee. The previous results are similar to the results of Lee *et al*., (2007) who reported that antioxidant enzymes were upregulated under heat stress in rice leaves, and also the enzymes related to metabolic pathway were differently accumulated.

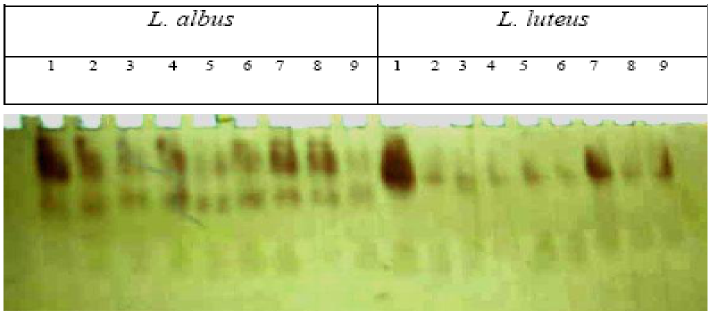

Fig. 4: Electrophoretic patterns in *L. albus and L. luteus* for polyphenol oxidase isozymes under control and different temperatures treatments. Lane 1=25°C (control), lane 2= 30°C/30 min, lane 3= 35°C/30 min, lane 4= 40°C/30 min, lane 5= 42°C/30 min and lanes 6, 7, 8 and 9= 42°C for1, 2, 3 and 4 h, respectively.

On the other hand, the highest activity from POX and PPO isozymes were found in *L. albus* exposed of 42ºC for 2 and 3 h, however the lowest isozyme activity at 42ºC for 4 h. Moreover, the maximum activity of POX and PPO isozymes scored into *L. luteus* plants under temperature 42ºC for 2 h and the control, while the minimum isozyme activity appeared in other treatments (Figs. 3 and 4 and Table 3). These results were in an agreement with Nagesh and Devaraj (2008) who confirmed that quantitative and qualitative alteration in antioxidant enzyme system are often related to level of resistance to stress, with quantitative changes in the enzyme level alterations were also observed in intensities and number of isozyme bands during applied stress. Like the present results, the decrease in isozyme activity indicates gradual degradation of these enzymes or their structural modification under increased Fe-toxicity levels, whereas the banding pattern expressing differential intensity shows the varying status of an enzyme affected by the stress.

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