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Original Research Paper

In Vivo and *In Vitro* effect of Hydroxycinnamic acids, IAA and Boron on the activity of Indole-3-actic acid Oxidase in Mung bean (Phaseolus aureus Roxb.) Cuttings

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Abstract: The In Vivo effect of *o*- and *p*- Hydroxy cinnamic acid and Caffeic acid when applied for cuttings at it's optimal concentration (10^{-3}M) and when applied each of them alone with simultaneous application with plant hormone IAA at it's optimal concentration (5×10^{-4} M) were investigated on the activity of the enzyme IAA-Oxidase (IAAO) and on the concentration of endogenous free hormone (IAA) in different parts and physiological periods on cuttings taken from 10-day-old seedlings grown in Deionized water (d\d H_2O) and also for that taken from 10-day-old seedlings grown in Boric acid (5µg/ml). However, The activity of IAAO was declined in cuttings were taken from seedlings grown in boric acid (5µg\ml) compared to d\d H_2O after 72 h. and the concentration of endogenous free hormone (IAA) was significantly increased in the hypocotyls of mung bean cuttings after 24h of basipetal application by the treatments in each case of cuttings taken from seedlings grown in d\d H_2O or boric acid(5µg\ml) compared to the control. The enzyme IAAO was extracted from hypocotyls of control cuttings after 72h from transferring it to the rooting medium which taken from seedlings grown in Deionized water (A) ,as well as for control cuttings taken from seedlings grown in boric acid (5µg/ml) (B) and *In Vitro* reacted with the following phenolic compounds (*o*-coumaric acid, *p*-coumaric acid , Caffeic acid) at the optimal concentration (10^{-3}M) and with boric acid (5µg/ml) , the hormone IAA (5×10^{-4}M) and with combination of each other, then the results by the means of enzymatic activity was compared with the same reactions carried out with the standard enzyme Horseradish peroxidase (HRP). The comparison pointed out for the role of IAAO as allosteric enzyme and suggested the effect of boron as (Negative / Positive) heterotropic allosteric effector.

Keywords: Hydroxy cinnamic, Caffeic acid, IAA-Oxidase, *p*-coumaric acid .

1. Introduction

The effect of *o*- and *p*- Hydroxy cinnamic acid and Caffeic acid on rooting response of mung bean cuttings with respect to auxin (IAA) & boron has been studied by (1) , they were reported that The effective concentration is 10^{-3}M for all of the above tested compounds, whether cuttings were taken from 10 –day-old light grown seedlings in deionized H_2O or in boric acid (5µg\ml). It has been shown that divisions of the first root initial cells are dependent upon either applied or endogenous auxins (2, 3). The endogenous levels of IAA decreased during Adventitious root formation (ARF) specially at the growth and development phase in which the root initials converted into visible roots (4). The regulation of plant development by IAA may depend on the amount of free IAA in plant tissue (5). Fee IAA can be modulated via several pathways, including IAA metabolism, synthesis and transport, and the breakdown of conjugated IAA. One well Known IAA metabolic pathway consists of the oxidative decarboxylation of the side chain of IAA by IAA-Oxidase (6 , 7). IAA-Oxidase is one of the enzymes which are associated with metabolism of endogenous IAA and regulation of it's concentration in

cutting during adventitious root formation, where found that activity of IAA-Oxidase declined during the first 20-40h in order to accumulation of IAA in hypocotyls of mung bean cutting to initiate the cell division and it's continuation until formation of root primordia (during initiation phase) , after that it's activity increased in object to reducing IAA level to initiate the growth and development phase , that include the conversion of root primordia into visible root (8 ; 9). It has been suggested from (10) that phenolics protect auxins from decarboxylation so that after application of phenolics more auxin are available to induce roots . It has been suggested from Shaheed (1987) that in order to the growth and development of root primordia to begin the levels of auxin must be decreased and that may be occur from the postulated role of boron in increasing the activity of IAA-Oxidase after the binding of Boron with Orthodiphenols compounds that act as auxin protectors . So it has been suggested again from Shaheed (1987) " in the presence of Boron in the form of Borate BO_3^{-3} it will be probably formation of complexes from Borate and di-hydroxyl phenolic compounds in *ortho* position causes enhancement in activity in IAA-Oxidase activity and the diminished concentrations of IAA resulted from that effect allows the subsequent growth and development of roots primordia . In addition to what previously mentioned the present paper suggests direct effect of Boron on the activity of IAA-Oxidase as heterotropic (Negative / Positive) allosteric effector and suggests antioxidants role for phenolic compounds tested as well as the possibility of bindig these compounds for Boron as a result of that an effect on the activity of IAA-Oxidase and the levels of endogenous free IAA. So the research aims to study the possibility of the existence of two active sites on the enzyme .

2. Methodology

Determination of IAAO activity and IAA levels in different physiological periods & parts Estimation of IAA Oxidase activity

IAA oxidase activity was estimated according to the method of Beffa *et al*. (1990) The standard reaction mixture contained in a final volume of 1 mL: 6.66 mm phosphate buffer (KH2PO4/Na2HPO4) at pH 6.00 ± 0.05, 100 µM $MnC1_2$, 50 µM *p*-coumaric acid, 85.6 µM IAA , and 200 µL of the extract tested. Assays were conducted at 24.0 ± 0.5°C for 30 min with shaking (200 rpm). The Salkowski reagent [1ml $FeCl_3$ + 50 ml d/H_2O + 30 ml H_2SO_4] (2 mL), was then added and the destruction of IAA was determined by measuring the absorbance at 530 nm after 30 min. One unit of IAAO activity was expressed in terms of µg of IAA oxidized/g fresh weight/30 min.

Estimation of horseradish peroxidase (HRP) activity

The activity of HRP was estimated according to the method of (11) in which the activity of HRP measured depending on the Colorimetric analyses of indoleacetic acid (IAA) oxidation reaction mixtures were made with the DMACA reagent (*p*-dimethylaminocinnamaldehyde) DMACA reagent will yield a wine-red color with IAA oxidation products in solution. determined by measuring the absorbance at 562 nm after 70 min.

Estimation of Protein

The protein content was estimated according to the method of Lowry *et al.* (1951) using crystallilne bovine serum albumin as the reference protein.

Estimation the levels of IAA

The IAA extraction and it's Spectrophotometric assay at 280 nm were done according to the method of Ergün *et al*. (2002).

Reaction of the HRP , IAAO in the case of (A) and IAAO in the case of (B) with Hydroxycinnamic acids , Boric acid and the substrate IAA within fourteen probability .

1) HRP, IAAO (A) and IAAO(B) + *o*-Coumaric acid.
2) HRP, IAAO (A) and IAAO(B) + *p*-Coumaric acid.
3) HRP, IAAO (A) and IAAO(B) + Caffeic acid.
4) HRP, IAAO (A) and IAAO(B) + Boric acid+ *o*-Coumaric acid.
5) HRP, IAAO (A) and IAAO(B) + Boric acid+ *p*-Coumaric acid.
6) HRP, IAAO (A) and IAAO(B) + Boric acid+ Caffeic acid.
7) HRP, IAAO (A) and IAAO(B) + Boric acid.

8) HRP, IAAO (A) and IAAO(B) + Boric acid+ *o*-Coumaric acid + $IAA_{Optimal}$.
9) HRP, IAAO (A) and IAAO(B)+ Boric acid+ *p*-Coumaric acid + $IAA_{Optimal}$.
10) HRP, IAAO (A) and IAAO(B) + Boric acid+ Caffeic acid + $IAA_{Optimal}$.
11) HRP, IAAO (A) and IAAO(B) + Boric acid + $IAA_{Optimal}$.
12) HRP, IAAO (A) and IAAO(B) + *o*-Coumaric acid + $IAA_{Optimal.}$
13) HRP, IAAO (A) and IAAO(B)+ *p*-Coumaric acid + $IAA_{Optimal}$.
14) HRP, IAAO (A) and IAAO(B) + Caffeic acid + $IAA_{Optimal}$.

Since (*o, p* – Coumaric acid and caffeic acid) was at concentration equal to 10^{-3}M and the optimal concentration of IAA was 5×10^{-4}M and the concentration of Boric acid was (5µ/ml) . The method of measuring activity was done according to the (7) and (Meudt and Gaines , 1967) except of the modification of incorporate the Boric acid and use high concentration of

hydroxycinnamic acid and IAA , in addition to the halving the amount of volume used when incorporate these compound to the reaction assay of activity .

3. Results and discussion

In Vivo effect of Hydoxycinnamic acids (*Ortho, Para* – Coumaric acids and Caffeic acid) on the activity of IAA-Oxidase and the level of free IAA in the mung bean cuttings .

Tables 1 & 2 Show the effect of basipetal application of the mung bean cuttings were taken from seedlings 10-old-days grown in Deionized water (case A) or in Boric acid (5µg/ml) (case B) with (*Ortho, Para* – Coumaric acids and Caffeic acid) each of them alone and at their optimal concentration (10^{-3}M) on the activity of IAA-Oxidase and the level of free IAA were extracted from epicotyls of mung bean cuttings at periods (0 , 24 , 72) h and as a result reflection of that effect on the rooting response . Whereas Tables 3 & 4 Show the effect of basipetal application of the mung bean cuttings in the cases of (A) & (B) with (*Ortho, Para* – Coumaric acids and Caffeic acid) at their optimal concentration (10^{-3}M) in simultaneous application with IAA at it's optimal concentration (5×10^{-4}M) on the activity of IAA-Oxidase and the level of free IAA were extracted from hypocotyls of mung bean cuttings at periods (0 , 24 , 72) h and as a result reflection of that effect on the rooting response . As the effect of these compounds when applied individual or combined with IAA as antioxidants illustrates from their inhibition for the activity of IAA-Oxidase leading to the increment in the level of free IAA and it's accumulation and as a result high rooting response in comparison with the rooting response of control in each case of cuttings (A) & (B) . Where in the case of (A) cuttings tables 1 & 3 the results revealed that the individual hydroxycinnamic acids and combined with IAA inhibited the activity of IAA-Oxidase in comparison to the control and corresponding to that significant increment on probability level (0.05) in the level of concentration of IAA that after 24h from basipetal application of cuttings with treatments , as well as significant increment on probability (0.01) was observed in IAA-Oxidase activity after 72h from transferring the cuttings into the rooting medium (Boric acid, 5µg/ml) . Whereas in the case of (B) cuttings tables 2 & 4 the same thing as above except that no significant increment on probability levels (0.05) or (0.01) was observed after 72h from transferring the cuttings into the rooting medium (d/dH_2O) . However, the activity of IAA-Oxidase was declined in cuttings were taken from seedlings grown for 10 days in boric acid (5µg/ml) [their rooting medium (d/H2O)] compared to that grown in d/dH_2O [their rooting medium boric acid 5µg/ml] that after 72 h of transferring it into rooting medium that belong to the role of supplied borate for 3 days (72 h) as rooting medium in the case of A cuttings and it's effect on increasing the activity of IAA-Oxidase compared to B cuttings that treated with (d/H_2O) as rooting medium . On the other hand and from table 5 the concentration of endogenous free hormone IAA was significantly increased on probability level (0.01) in the hypocotyls of mung bean cuttings after 24h of basipetal application by treatments hydroxycinnamic acid above mentioned in each case of cuttings (A & B).

Table 1: Influence of hydroxycinnamic acids on IAAO activity[*µg IAA Oxidized/30min/g fresh tissue*] and concentration of free IAA (M.) in mung bean epicotyls cuttings taken from seedlings grown in Deionized water (A).

Treatments	Activity of IAA-Oxidase *µg IAA Oxidized/30min/g fresh tissue* Epicotyls Sections			Concentration of IAA (M.) Epicotyls Sections			Mean root No./ cutting
	Immediately After cuttings were taken	24 h	72 h	Immediately After cuttings were taken	24 h	72 h	
The Control	0.433	0.360 ↓	1.215	1×10^{-3}	9.5×10^{-4} ↑	9.5×10^{-4}	6.6
10^{-3} *M Caffeic acid*	.....	0.355 (%1.4)	1.067	.....	1.9×10^{-3} (%100)	1.7×10^{-3}	20.3
10^{-3} *M p*-Coumaric acid	.....	0.352 (%2.2)	1.101	.....	2.6×10^{-3} (%173.6)	2.3×10^{-3}	21.7
10^{-3} *M o*-Coumaric acid	.....	0.344 (%4.5)	1.109	.....	5×10^{-3} (%426.3)	4.1×10^{-3}	22.8

LSD for IAAO activity Column LSD(0.05) = 0.6 ; LSD(0.01) = 1.2
LSD for IAAO activity Rows LSD(0.05) = 0.1 ; LSD(0.01) = 0.2
LSD for IAAO activity Interaction LSD(0.05) = 0.029 ; LSD(0.01) = 0.27
LSD for IAA Conc Columns LSD(0.05) = 4.5×10^{-3} ; LSD(0.01) = 8.2×10^{-3}
LSD for IAA Conc Rows LSD(0.05) = 4.6×10^{-3} ; LSD(0.01) = 8.5×10^{-3}
LSD for IAA Conc Interactions LSD(0.05) = 2.3×10^{-3} ; LSD(0.01) = 4.3×10^{-3}

Table 2: Influence of hydroxycinnamic acids on IAAO activity[*µg IAA Oxidized/30min/g fresh tissue*] and concentration of free IAA (M.) in mung bean epicotyls cuttings taken from seedlings grown in Boric acid (5µg/ml) (B).

Treatments	Activity of IAA-Oxidase *µg IAA Oxidized/30min/g fresh tissue* Epicotyls Sections			Concentration of IAA (M.) Epicotyls Sections			Mean root No./ cutting
	Immediately After cuttings were taken	24 h	72 h	Immediately After cuttings were taken	24 h	72 h	
The Control	0.477 ↓	0.393	0.436	1.2×10^{-3}	1.2×10^{-3} ↑	1.7×10^{-3}	6.3
10^{-3} *M Caffeic acid*	.....	0.414(13.2%)	0.434	.....	1.4×10^{-3} (%16.6)	2.8×10^{-3}	18.8
10^{-3} *M p*-Coumaric acid	.....	0.404 (15.3%)	0.395	.....	1.8×10^{-3} (%50)	3×10^{-3}	17.3
10^{-3} *M o*-Coumaric acid	.....	0.388(18.6%)	0.368	.....	1.9×10^{-3} (%58.3)	2.9×10^{-3}	17.3

LSD for IAAO activity Column LSD(0.05) = 0.5 ; LSD(0.01) = 0.92
LSD for IAAO activity Rows LSD(0.05) = 0.49 ; LSD(0.01) = 0.91
LSD for IAAO activity Interaction LSD(0.05) = 0.51 ; LSD(0.01) = 0.94
LSD for IAA Conc Columns LSD(0.05) = 3.7×10^{-3} ; LSD(0.01) = 6.9×10^{-3}
LSD for IAA Conc Rows LSD(0.05) = 1.3×10^{-3} ; LSD(0.01) = 2.4×10^{-3}
LSD for IAA Conc Interactions LSD(0.05) = 8.3×10^{-4} ; LSD(0.01) = 1.5×10^{-3}

Table 3: Influence of combination of hydroxycinnamic acids with IAA on IAAO activity[*µg IAA Oxidized/30min/g fresh tissue*] and concentration of free IAA (M.) in mung bean epicotyls cuttings taken from seedlings grown in Deionized water (A).

Treatments	Activity of IAA-Oxidase *µg IAA Oxidized/30min/g fresh tissue* Hypocotyls Sections			Concentration of IAA (M.) Hypocotyls Sections			Mean root No./ cutting
	Immediately After cuttings were taken	24 h	72 h	Immediately After cuttings were taken	24 h	72 h	
The Control	0.393	0.363 ↓	1.049	5.7×10^{-4}	8.9×10^{-4} ↑	1.2×10^{-3}	6.2
10^{-3} *M Caffeic acid + (5×10^{-4} M) IAA*	.....	0.341 (6.1%)	1.152	.....	2.7×10^{-3}(%203.3)	2.1×10^{-3}	39.3
10^{-3} *M p*-Coumaric acid+ *(5×10^{-4} M) IAA*	.....	0.354 (2.5 %)	1.123	.....	2.4×10^{-3}(%169.6)	1.9×10^{-3}	29.5
10^{-3} *M o*-Coumaric acid+ *(5×10^{-4} M) IAA*	.....	0.361 (0.6%)	1.078	.....	2.2×10^{-3}(%147.1)	1.6×10^{-3}	24

LSD for IAAO activity Column LSD(0.05) = 2.1 ; LSD(0.01) = 4.0
LSD for IAAO activity Rows LSD(0.05) = 0.05 ; LSD(0.01) = 0.09
LSD for IAAO activity Interaction LSD(0.05) = 0.073 ; LSD(0.01) = 0.13
LSD for IAA Conc. Columns LSD(0.05) = 4×10^{-3} ; LSD(0.01) = 7.3×10^{-3}
LSD for IAA Conc. Rows LSD(0.05) = 1.7×10^{-3} ; LSD(0.01) = 3.23×10^{-3}
LSD for IAA Conc. Interactions LSD(0.05) = 1×10^{-3} ; LSD(0.01) = 1.9×10^{-3}

Table 4: Influence of combination of hydroxycinnamic acids with IAA on IAAO activity[*µg IAA Oxidized/30min/g fresh tissue*] and concentration of free IAA (M.) in mung bean epicotyls cuttings taken from seedlings grown in Boric acid (5µg/ml) (B).

Treatments	Activity of IAA-Oxidase *µg IAA Oxidized/30min/g fresh tissue* Hypocotyls Sections			Concentration of IAA (M.) Hypocotyls Sections			Mean root No./ cutting
	Immediately After cuttings were taken	24 h	72 h	Immediately After cuttings were taken	24 h	72 h	
The Control	0.444 ↓	0.370	0.444	1.2×10^{-4}	1.9×10^{-3} ↑	2.7×10^{-3}	5
10^{-3} *M Caffeic acid + (5×10^{-4} M) IAA*	.....	0.328(26.1%)↓	0.356	.....	3.1×10^{-3}(%63.1)	2.3×10^{-3}	29.9
10^{-3} *M p*-Coumaric acid+ *(5×10^{-4} M) IAA*	.....	0.395 (11%)↓	0.445	.....	2.8×10^{-3}(%47.3)	2.1×10^{-3}	18.7
10^{-3} *M o*-Coumaric acid+ *(5×10^{-4} M) IAA*	.....	0.460 (3.6%)↑	0.478	.....	2.4×10^{-3}(%26.3)	1.6×10^{-3}	19.7

LSD for IAAO activity Column LSD(0.05) = 0.51 ; LSD(0.01) = 0.94
LSD for IAAO activity Rows LSD(0.05) = 0.57 ; LSD(0.01) = 1.06
LSD for IAAO activity Interaction LSD(0.05) = 0.46 ; LSD(0.01) = 0.85
LSD for IAA Conc. Columns LSD(0.05) = 6.7×10^{-3} ; LSD(0.01) = 0.012
LSD for IAA Conc. Rows LSD(0.05) = 8.7×10^{-4} ; LSD(0.01) = 1.6×10^{-3}
LSD for IAA Conc. Interactions LSD(0.05) = 1.1×10^{-3} ; LSD(0.01) = 2×10^{-3}

Table 5: Influence of hydroxycinnamic acids on the concentration of free IAA (M.) in mung bean epicotyls cuttings taken from seedlings grown in Deionized water (A) or in Boric acid (5µg/ml) (B).

Treatments	Concentration of IAA (M.)					
	A Hypocotyls Sections			B Hypocotyls Sections		
	Immediately After cuttings were taken	24 h	72 h	Immediately After cuttings were taken	24 h	72 h
The Control	5.6×10^{-4}	9.5×10^{-4} ↑	1.2×10^{-3}	1.2×10^{-3}	1.9×10^{-3} ↑	2.7×10^{-3}
10^{-3} *M Caffeic acid*	.....	2.8×10^{-3}(%194.7)	3.4×10^{-3}	.....	2.3×10^{-3}(%21)	2.8×10^{-3}
10^{-3} *M p*-Coumaric acid	.....	3.6×10^{-3}(%278.9)	4.4×10^{-3}	.....	2.5×10^{-3}(%31.5)	2.6×10^{-3}
10^{-3} *M o*-Coumaric acid	.....	5.2×10^{-3}(%447)	2.3×10^{-3}	.....	2.9×10^{-3}(%52.6)	2.8×10^{-3}

A LSD for IAA Conc. Columns LSD(0.05) = 7.3×10^{-3} ; LSD(0.01) = 0.013
LSD for IAA Conc. Rows LSD(0.05) = 3.9×10^{-4} ; LSD(0.01) = 7.2×10^{-4}
LSD for IAA Conc. Interactions LSD(0.05) = 2.9×10^{-4} ; LSD(0.01) = 5.4×10^{-4}
B LSD for IAA Conc. Columns LSD(0.05) = 4.1×10^{-3} ; LSD(0.01) = 7.6×10^{-3}
LSD for IAA Conc. Rows LSD(0.05) = 6.7×10^{-4} ; LSD(0.01) = 1.2×10^{-3}
LSD for IAA Conc. Interactions LSD(0.05) = 6.2×10^{-4} ; LSD(0.01) = 1.1×10^{-3}

A free radical mechanism was proposed for the oxidation of IAA by IAA-Oxidase (14). The generally accepted theory is that phenolic inhibitor reducing the oxidation of IAA by removing the H_2O_2 that formed in the oxidative cycle and the phenolic inhibitors trap free radical intermediates which would otherwise contribute to the oxidation of IAA (15 ; 16). The trapping of free radical (that formed in the oxidative cycle of IAA destruction) by phenolic inhibitors in which hydroxycinnamic acid depending on what these compounds visualized as antioxidants depending on their chemical structure which provides ability and conjugation area to ampleness the free radicals that formed in the oxidative cycle of IAA destruction and by that inhibit the oxidation that because the presence of high electronic conjugation in those phenolics in comparison with other antioxidants (17 ; 18) acting their role as protectors of auxin from oxidizing it via IAA-Oxidase. Where the presence of OH group in the ortho position according to substituted group in *o*-Coumaric acid was as activator of ring since the function of this position is to push electrons (electron donor) and hence, leads to increase the active electronic conjugation to include the area of OH, ring and side group in the direction of COOH group . This explanation gave strong character for o-Coumaric acid as anti-oxidant and as a result more ability for the movement of free radical of $IAA^{\bullet+}$ in it's conjugation area compared to caffeic acid which characterized with two (OH) groups in *meta* and *para* positions distinguishable feature of caffeic acid. The above positions permits *intra* hydrogen bonding,

that leads to restrict the ionization of one of these (OH) groups in these positions .

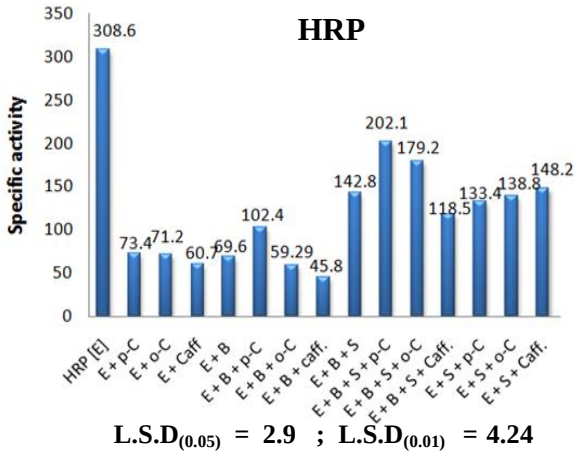
The caffeic acid has the same acidity and electronic conjugation as was the case in *o*-Coumaric acid . Whereas in the second case of caffeic acid ionization :

Caffeic acid has a position that carry oxygen with negative charge in *meta* position, which weakened the ring and is considered as inactivate for electronic conjugation as a result a weakened character for this case as anti-oxidant . However, *p*-Coumaric acid considered midway in acidity and electronic conjugation between o-Coumaric acid and caffeic acid as antioxidant since it has only one (OH) group in *para* position according to the substituted group .

On the other hand , the occurrence of reflux antioxidant effect for hydroxycinnamic acids after their binding each of them alone with IAA in case of combination, as the presence of electronic conjugation area in that compounds which have chemical structure allowed to them with what contain of hydrogen groups to form binding with IAA to provides conjugation area include on each of the two bounding molecules . So the effect of hydroxycinnamc acid in their cases alone and combined with IAA were clearly illustrated in this study by the inhibition of the activity of IAA-Oxidase and the level of the Phytohormone IAA and as a result of that rise in rooting response as was observed in tables 3 & 4 . Since one of the hydroxyl groups n caffeic acid allowed formation binding with IAA acquired by that more acidity for the compound resulted from binding (IAA-caffeic acid) and greater electronic conjugation area in which that make it strong antioxidant and scavenger for electrons and good inhibitor for oxidation cycle and as a result higher free IAA level and higher rooting response. The combination of [IAA – Caffeic acid] proposed from (19) under the effect of enzymes IAA-Oxidase and Polyphenoloxidase *in vivo* .

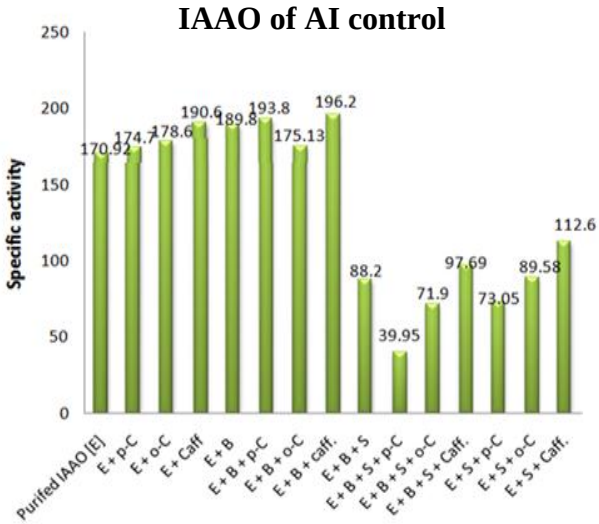
The combination of [IAA – Caffeic acid] has more acidity than [IAA – *o*-Coumaric acid] and the last has more acidity than[IAA – *p*-Coumaric acid] this can explain why the inhibition of IAA-Oxidase and as a result increasing n the IAA level and rooting response have been according to the size of electronic conjugation area formed as follow [IAA – Caffeic acid] > [IAA – *p*-Coumaric acid] > [IAA – *o*-Coumaric acid] the opposite occur when applied Hydroxycinnamic acids alone as the size of electronic conjugation area inversed as follow [*o*-Coumaric acid] > [*p*-Coumaric acid] > [Caffeic acid]

The Reaction of the Standard enzyme Horseradish peroxidase (HRP) and the extracted enzyme Indole-3-acetic acid Oxidase (IAA-Oxidase) from the controls (AI) & (BI) with Hydroxycinnamic acids' derivatives, Boric acid and Indole-3-acetic acid.



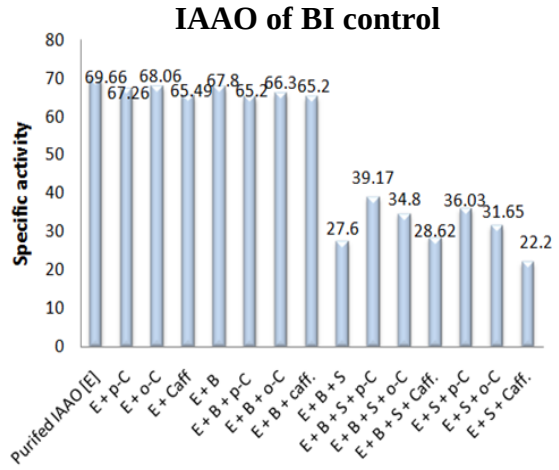
L.S.D$_{(0.05)}$ = 2.9 ; L.S.D$_{(0.01)}$ = 4.24

Figure 4: In Vitro Reactions of HRP with Hydroxycinnamic acids' derivatives, Boric acid and Indole-3-acetic acid



L.S.D$_{(0.05)}$ = 3 ; L.S.D$_{(0.01)}$ = 4.4

Figure 5: In Vitro Reactions of IAAO of AI control with Hydroxycinnamic acids' derivatives, Boric acid and Indole-3-acetic acid



L.S.D$_{(0.05)}$ = 2.8 ; L.S.D$_{(0.01)}$ = 4.3

Figure 6: In Vitro Reactions of IAAO of BI control with Hydroxycinnamic acids' derivatives, Boric acid and Indole-3-acetic acid

Generally, it has been appeared from the two figures 4 & 6 that the reactions of standard enzyme HRP were in accordance with *in vitro* reactions of the enzyme IAA-Oxidase that purified from BI control and that agreement in the side of significant inhibition of the enzymatic activity on probability level (0.05) by the hydroxycinnamic acids (caffeic acid , *o*-coumaric acid , *p*-coumaric acid) each of them alone at their optimal concentration for rooting response (10^{-3}M) and also inhibition the enzymatic activity that observed when the reaction with boric acid (5µg/ml) and when the reaction with combination formed from [boric acid + hydroxycinnamc acid] , [boric acid + IAA + hydroxycinnamc acid] and [IAA + hydroxycinnamc acid] with the observation of varying ratios of inhibition in each case of reaction between two figures 4 & 6 .However, this agreement can be interpreted through the fact that each of enzymes HRP and IAA-Oxidase from BI control behave in low affinity towards the substrate IAA in spite of the enzyme IAA-Oxidase purified from BI control in which it's cuttings taken from seedlings 10-old-day grown in boric acid (5µg/ml) but it's rooting medium was (d/H_2O) that because the boron in the form of borate that applied for 10 days to germinate the seedlings activate the enzyme in the allosteric site and because of the length of duration of borate effect on the enzyme and the occurrence of saturation for enzyme by the borate concentration in the allosteric site that lead to strong binding of borate to the IAA-Oxidase in which the borate playing it's role as Heterotropic negative allosteric effector leads to structural conformation n enzyme modifying by that the active site by acquired it the (tight conformational form) the form that correlated with low affinity of enzyme for it's substrate (20). So the enzyme IAA-Oxidase from BI control has low affinity for IAA as well as with the enzyme HRP that doesn't contain borate. Whereas the figure 5 characterized with the *in vitro* reactions that carried out with IAA-Oxidase from AI control have been led to significant increasing in enzyme activity on the probability (0.01) in the case of reactions that don't have the substrate IAA in the (5×10^{-4}M) , as the role of boron in the form of borate that applied for 3 days (72h) as rooting medium after the first (24h) from taking the cuttings in increasing the activity of IAA-Oxidase and that belong to the suggested role of boron in increasing the activity of IAA-Oxidase from (Shaheed, 1987) that in pre accordance with (21 ; 22) . Since the comparison between the two cases , the first case the two figures 4 & 6 and the second case figure 5 gave clear indication to 1) the allosteric nature of the enzyme IAA-Oxidase where this allosterical nature of IAA-Oxidase foresaid and introduced from (23) in which the enzyme has two sites one of them active and the other allosteric . 2) the behavior of boron in the form of borate as (Heterotropic positive allosteric effector) , since the enzyme IAA-Oxidase considered as glycosylated enzyme videlicet, containing glycosidic group and also the presence of fractions in the proximal domain of tertiary structure for plant peroxidase being apart from the active center and extensively glycosylated similar to what HRP molecule contain of three oligosaccharide chains in the same proximal domain of HRP protein (24) . However, the glycoside group in the IAA-Oxidase acts it's role as active allosteric site to which the activate molecule for enzyme activity binds that called (Activator effector) that increase the affinity of enzyme for it's substrate , the activator effector represented by the borate molecule , this borate nearly binds to *cis*-hydroxyl pairs groups , these hydroxyl pairs specially present in carbohydrate and o-diphenols (25). The binding of borate as (Heterotropic positive allosteric effector) to the allosteric site in the IAA-Oxidase be formed though the formation of complexes between borate and hydroxyl groups that belong to glycosylated moiety leading to modification in the tertiary structure of enzyme that affect on the active site approving by that the affinity of IAA-Oxidase towards it's substrate IAA by acquired it the (relaxed conformational form) the form that correlated with high activity of enzyme (20) and that when applied borate for 3 days (72h) as rooting medium after the first (24h) from taking the cuttings . Since it is well known that the role of glycosylated moiety in the glycoenzyme was the maintenance of tertiary structure of enzyme. The structural conformations that made it the borate by forming complexes in the allosteric site was kind of positive cooperative action that occur at the

presence of multi binding sites on the protein molecule that the interaction may occur between these binding sites .Videlicet, the binding of one ligand will increase the affinity of subsequent site on the protein for other different ligand (26). Finally from all above introduced we can conclude that the borate affect the enzyme IAA-Oxidase directly in the two cases of controls AI & BI the varying in it's effect depending on the duration of application it and the occurrence of saturation for the IAA-Oxidase by it or not . While the effects of hydroxycinnamic acids alone and when present in combination with IAA as antioxidants depending on their chemical structures were on the activity of IAA-Oxidase that affected by borate . However , the effect of borate as (negative / positive heterotropic allosteric effector) on the activity of IAA-Oxidase has been approved in *the vivo* from tables (1, 2, 3, 4, 6, 7) also from figures (1, 2, 3) and in the vitro from the comparison between figures (4, 5, 6) . Whereas the effect of hydroxycinnamic acids alone and when present in combination with IAA as antioxidants depending on their chemical structures on the activity of IAA-Oxidase has been approved in *the vivo* but disproof in *the vitro* that can be interpreted to presence of the enzyme Polyphenol Oxidase (PPO) in the interior of plant cells that has a role in the combination of hydroxycinnamic acids with IAA in *the vivo* (19) and may be not presence of which when the *in vitro* reactions carried out , while the effect of Hydroxycinnamic acids alone as antioxidants depending on their chemical structures on the activity of IAA-Oxidase in *the vivo* because the probability of presence of other factors in the plant cells made the depending of these molecules on their chemical structures in inhibition of IAA-Oxidase activity.

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