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Phenolic acid analysis in Indian wheat cultivar exposed to drought stress

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Abstract
Drought stress diminishes growth and development which results in reduced production and yield of crop plants that are economically valuable. It is important to understand the adaptation behaviour of the crop plant against drought-prone environment as well as capabilities that help in the survival of the plant under unfavourable climatic conditions. The aim of this study was to evaluate the wheat germplasm at phytochemical level under imposed drought stress. For this purpose 20 wheat genotypes were treated with different concentration of PEG to imposed drought condition. After the present study it was observed that a progressive induced water stress using PEG-6000 causes significant changes in phenolic acid production in wheat. The genotype HD2733 and K9107 seem to be promising for detection and presence of high number of phenolic acids under drought stress.

Keywords: Abiotic stress, drought, high-performance liquid chromatography, phenolic acids, secondary metabolites and thin-layer chromatography.

Introduction
Biochemical elements and phytochemical compounds that show antioxidant activity within plants are proved to provide inert defense and tolerance. Wheat crop synthesized those secondary metabolites which protect it under drought stress (Qayyum *et al.* 2017) [8]. Secondary metabolites are naturally synthesized compounds catalysing various physiological functions (Chen *et al.* 2009) [2]. Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and industrially important biochemical. Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions. Environmental factors *viz.* temperature, humidity, light intensity, water, minerals and CO_2 influence the growth of a plant and secondary metabolite production. Drought, high salinity and freezing temperatures are environmental conditions that cause adverse effects on the growth of plants and the productivity of crops (Ramakrishna 2011) [10]. The stressed condition acts as stimulus to plants for regulating secondary metabolites, which are utilized in defense mechanism and stress tolerance (Zhao *et al.* 2005) [20]. Flavonoids are a group of phenolic that consist of two aromatic rings linked by three carbons. At present, more than 9000 different flavonoids have been discovered due to varieties of modification reactions in flavonoids biosynthesis (Ververidis *et al.* 2007) [16]. Despite their diversity of functions and structures, all flavonoids are derived from the general phenylpropanoid pathway, one of the best-known pathways in plant secondary metabolism. Flavonoids have protective functions under drought stress. They also provide protection against UV, pathogens, pest (Winkel-Shirley 2001) [18]. Many structural genes are involved in flavonoid biosynthetic pathway that give rise to many alternate branches producing various precursors, intermediates, flavonoid-like and other compounds (Pollastri and Tattini 2011) [7]. The flavonoid pathway becomes complex according to the stages and intensity of the stress (Shan *et al.* 2009) [14]. Flavonoid defensive factor against environmental stresses made it centre of attraction for research purposes. It was found that under drought condition the total phenolic, flavonoid, anthocyanin, schaftoside content accumulation increases in wheat crop. Similarly, the level of gene expression of genes involved in flavonoid biosynthesis enhanced in wheat under drought stress (Ma *et al.* 2014) [4].

Phytochemical characters and drought
Phenolic compounds such as phenolic acids and flavonoids are major plant secondary metabolites that are produced from the shikimate-phenylproponoid biosynthetic pathway. The stress plant produces a high level of phenolic compounds to prevent plant from oxidative stress

caused by ROS scavenging mechanism. Phenolic acids, flavonoids, condensed tannins, coumarins and alkyl-resorcinols are the examples of polyphenols. Phenolic acids are derivatives of benzoic and cinnamic acids. There are two classes of phenolic acids: hydroxybenzoic acid and hydroxycinnamic acid. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, vanillic, syringic and protocatechuic acids. Hydroxycinnamic acid coumaric, caffeic, ferulic and sinapic acids (Matilla *et al.* 2005) [5]. Free phenolic acids are located in the outer layer of pericarp and are extracted using organic solvents.

Abiotic stress induces oxidative damage in plant cells due to increased generation of noxious reactive oxygen species (ROS) in chloroplasts (Yildiz *et al.* 2009; Tian *et al.* 2004) [19,15]. Plants possess a number of phenolic compounds, and they have been proclaimed to be involved in oxidative stress caused by ROS (Wahid and Ghazanfar 2006) [17]. On the other hand, plants under certain stress conditions often produce a higher degree of phenolic compounds compared to non-stressed plants (Selmar 2008) [13].

Material and Method

Field Trial

The experiment was conducted at field laboratory of Department of Agriculture Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, U.P., during *rabi* season of 2015-16 and 2016-17. Rabi is the winter season in Northern India, where crop is sown in October-November and harvested in March-April. The cultivars are: K-802, K-1256, K-607, K-9107, K-6525, K-9423, DBW-71, DBW-16, DBW-17, MP-4010, MP-3336, PBW-226, PBW-373, PBW-590, PBW-71, PBW-533, HD-2733, HD-3086, HD-3095 and HD-2864. The seeds of all wheat genotypes were treated with three concentrations (10%, 20% and 30%) of Poly Ethylene glycol (PEG) for 48 hours in Petri plates in three replications along with control and allow them to germinate. After 48 hours, the seedlings were transferred to pots and field and maintained under optimum conditions till maturity. The Experiments was conducted under Randomized Block Design (RBD) throughout the study. In each block 25 seeds were spreaded randomly. Every replication was tagged with their genotype name and PEG concentration (10%, 20% and 30%). After transferring the seeds, the pots and field was only irrigated in excessively dry condition for maintaining the stress; otherwise it was depended on rain water. The crop was observed for phytochemical characterization by chromatography techniques.

Phytochemical characterization

Total phenolic content

100 mg leaf was crushed in 1ml methanol. 0.3ml filtered extract was added in 2ml of Folin-Ciocalteu reagent to which 1.6 ml of sodium carbonate was added. The mixture was incubated at room temperature for 2 hours. The total phenolic content was quantification by UV absorbance at 760nm.

Analysis of phenolic acids by thin layer chromatography

Extract was prepared by crushing 100mg of fresh leaf tissue in 1ml of methanol. Standard solutions for hydroxycinnamic acid i.e. *p*-&*o*-coumaric, caffeic, ferulic, sinapic acid and hydroxybenzoic acids: p-hydroxybenzoic, vanillic and salicylic acid were prepared (10 mg in 1ml methanol) and preserved at 4˚C for further use. Thin layer chromatography was performed on analytical plates of silica gel with differential solvent system of benzene: acetic acid: water in ratio of 37: 45: 18. TLC plates were air dried and sprayed with 2% ferric chloride solution for development of different spots/bands. The movement of the analyse was expressed by its retention factor (R*f*). Values were calculated for different sample.

$$\mathbf{R}f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Analysis of phenolic acids by HPLC

The extract prepared for TLC was diluted 10 times with methanol for analysis using HPLC. Standard solutions for hydroxycinnamic acid i.e. *p*-&*o*-coumaric, caffeic, ferulic, sinapic acid and hydroxybenzoic acids: p-hydroxybenzoic, vanillic and salicylic acid were prepared (10 mg in 1ml methanol). Chromatographic separation was performed on Shimadzu HPLC instrument with C18 column at flow rate 0.5 ml/min for 20 minutes. The solvent system used was water: acetonitrile (92:8). Measurements of phenolic compound concentrations were performed using an external standard at wavelengths λ = 280. Compounds were identified based on a comparison of retention times of the examined peak with that of the standard and by adding a specific amount of the standard to the tested sample and repeated analyses. Quantification of phenolic compounds was based on peak areas and calculated as equivalents of representative standard compounds.

Result

Phytochemical characterization

Total phenolic content

Quantitative analysis of total phenolic content in leaf extract of wheat genotypes under study was done by using UV/VIS Spectrophotometer (PerkinElmer UV/VIS spectrometer Lambda 25) (Table:1).

Table 1: Spectrophotometric analysis of total phenolic content of 20 wheat genotypes

Wheat Genotypes	C	T1	T2	T3
K1256	0.90	1.30	1.41	1.49
HD2733	0.95	1.21	1.33	1.76
HD3086	0.81	1.03	1.15	1.35
HD3095	0.80	0.89	0.97	1.25
DBW16	0.79	0.87	1.01	1.23
PBW533	0.85	0.93	1.19	1.32
PBW590	0.92	0.97	1.13	1.21
PBW71	0.75	0.89	0.97	1.07
HD2864	0.78	0.90	1.08	1.14
DBW71	0.86	0.93	1.03	1.15
PBW226	0.81	0.88	0.97	1.15
MP3336	0.74	0.86	0.93	0.99
K9107	0.93	1.18	1.24	1.36
PBW373	0.77	0.90	0.98	1.23
K607	0.75	0.88	0.97	1.05
DBW17	0.78	0.85	0.96	1.08
K6525	0.81	0.93	1.04	1.19
K802	0.79	0.87	0.97	1.15
MP4010	0.66	0.79	0.94	1.09
K9423	0.68	0.81	0.95	1.13

Thin layer chromatography (TLC)

Phenolic acids plays an important role in plants, undergoing stressed conditions. Their accumulation increases the stress tolerance ability of the plant. The methanolic extract of wheat

leaves sample was spotted on TLC plates against phenolic acid standards *viz*. p-coumaric, *o*-coumaric, caffeic, ferulic, sinapic acid, salicylic acid, p-hydroxybenzoic acid and vanillic acid were run in solvent system (Benzene : Acetic Acid : Water 37:45:18).

The standard of phenolic acids gives different colour when plates were developed with $FeCl_3$. The Rf value of all phenolic acids were calculated as ready reference for identity of phenolic acids in leaf extract of wheat (Table 2; Fig.1). The p-coumaric acid gives Dark yellow colour, Ferulic acid gives Orange colour, o-coumaric acid gives light yellow colour, caffeic acid gives green colour and sinapic acid gives pink colour after treating with $FeCl_3$. Similarly salicylic acid gives violet colour, p-hydroxybenzoic acid gives light yellow and vanillic acid gives light orange colour. All the taken phenolic acid standards shows different Rf value as p-coumaric acid at 0.38, ferulic acid at 0.62, o-coumaric acid at 0.43, caffeic acid at 0.12, sinapic acid at 0.53, salicylic acid at 0.97, p-hydroxybenzoic acid at 0.75 and vanillic acid at 0.81.The leaf methanolic extract of different genotypes wheat were also allowed to run in the same solvent system as in standards. Different wheat genotypes showed the presence of different compounds of phenolic acids. Of them only ferulic (Rf- 0.62; colour-orange), caffeic (Rf-0.12; colour- green) and p-hydroxybenzoic acid were identified with reference to standards in the leaves extract of wheat. The ferulic acid was observed into standards in the leaves extract of wheat. The ferulic acid was observed in genotype K1256, HD2733, HD3086, HD3095, DBW16, PBW533, PBW590, DBW71, K9107 and PBW373. However the genotype MP3336, K607 and PBW373. Whereas the genotype PBW71, K607 and K9423 shows the presence of ferulic acid only after imposing drought stress at T3 level of PEG treatment. The genotypes that showed the presence of both phenolic acids are comparatively useful under drought stress conditions. The genotypes HD2733, HD3086, HD3095, DBW16, PBW533, PBW590, DBW71, K9107 showed the presence of both ferulic and caffeic phenolic acids. The p-hydroxybenzoic acid was also observed in genotypes HD2733, DBW71, HD3086, DBW16, MP3336, K9107, PBW373, K607, K6525, K802, MP4010 and K9423. Two unidentified bands having Rf value .95 and .80 of light and dark green colour respectively were also observed in different genotypes.

High performance liquid chromatography (HPLC)

The variation in qualitative and quantitative distribution of tentatively identified caffeic acid, ferulic acid and other unidentified compounds in different wheat genotypes using TLC were further examined using more sophisticated and accurate technique as reverse phase HPLC (C-18 column). The HPLC was performed using binary solvent system as described earlier.

In order to identify more accurately the phenolic compounds, the standards of phenolic acid *viz*. p-coumaric, *o*-coumaric, caffeic, ferulic, sinapic acid, salicylic acid, p-hydroxybenzoic acid and vanillic acid were run independently and then co-chromatographed using solvent system A (2% glacial acetic acid in water) and solvent B (30% acetonitrile and 2% glacial acetic acid in water). The HPLC chromatograms of these standards are shown in figure 2.

The available standards of phenolic acids were separated and gave sharp and clear peaks at different retention time (RT). The RT of p-coumaric, *o*-coumaric, caffeic, ferulic, sinapic acid, salicylic acid, p-hydroxybenzoic acid and vanillic acid are shown in Table 3; Fig:2. Based on the results of TLC, six wheat genotypes which show the presence of phenolic acids were chromatographed. A comparison of RT of individual phenolic acid and co-chromatography of various standards showed, that HD2733 showed presence of p-hydroxybenzoic, caffeic, ferulic, sinapic acid and *o*-coumaric in control and treated plants, vanillic acid and salicylic acid showed presence in control and T1, whereas p-coumaric was absent in control and treated plants.

The genotype PBW533 showed presence of ferulic and caffeic in control and treated plants, whereas salicylic acid showed presence in T1,T2 and T3 only and vanillic acid in T1. The genotype DBW71 showed presence of ferulic (C,T1,T2,T3) and caffeic (C,T1,T2,T3), whereas p-hydroxybenzoic acid was present in T1 and T2; salicylic acid was present in T1 and T3 only.

The genotype K9107 showed presence of p-hydroxybenzoic acid, salicylic acid, ferulic and caffeic acid in control, T1, T2, T3. The genotype PBW373 showed the presence of p-hydroxybenzoic acid, salicylic acid, ferulic, sinapic acid and caffeic acid in control, T1, T2, T3. The genotype K6525 showed the presence of sinapic acid in control, T1and T2; Fig: 2 A,B,C.

Concentration of phenolic acids was calculated from peak areas as equivalents of representative standard compounds. (Table 4 A, B).

Table 2: Rf value of all phenolic acids

S. No.	Standards	Rf Value	Colour	Solvent System
1	p-coumaric	0.38	Dark yellow	Benzene: Acetic acid: Water (37:45:18)
2	Ferulic	0.62	Dark Orange	
3	o-coumaric	0.43	Light yellow	
4	Caffeic	0.12	Green	
5	Sinapic	0.53	Pink	
6	Salicylic Acid	0.97	Violet	
7	p-Hydroxybenzoic Acid	0.75	Light yellow	
8	Vanillic Acid	0.81	Light Orange	

Table 3: RT value of all phenolic acids

S.No.	Standards	RT Value	Solvent System
1	p-coumaric	4.375	Water : Acetonitrile (92:8)
2	Ferulic	7.538	
3	o-coumaric	4.537	
4	Caffeic	3.425	
5	Sinapic	4.258	
6	Salicylic Acid	8.279	
7	p-Hydroxybenzoic Acid	1.907	
8	Vanillic Acid	5.245	

Table 4: (A) Concentration of different Standards detected in Wheat Genotypes in HPLC

S.No.	Standard→ Genotypes↓	*p*-Hydroxy benzoic Acid (µg/ml)				Vanillic Acid (µg/ml)				Salicylic Acid (µg/ml)				Ferulic Acid (µg/ml)			
1.	HD2733	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		.016	.018	.021	.021	.051	.081	-	-	.085	.066	-	-	.020	.023	.024	.027
2.	PBW533	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		-	-	-	-	-	.336	-	-	-	.014	.025	.033	.001	.002	.010	.001
3.	DBW71	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		-	-	.013	-	-	-	-	-	-	.174	-	.079	.008	.099	.053	.118
4.	K9107	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		.010	.010	.013	.010	-	-	-	-	.078	.078	.283	.293	.011	.022	.023	.047
5.	PBW373	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		.009	.010	.016	.016	-	-	-	-	.139	.160	.175	.190	.050	.052	.121	.131
6.	K6525	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		-	-	-	-	-	-	-	-	.022	.024	.024	.026	-	-	-	-

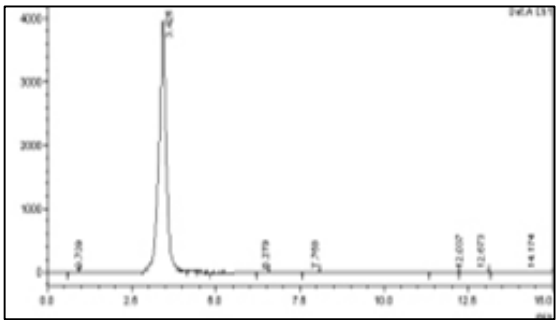
Table 4 (B): Concentration of different Standards detected in Wheat Genotypes in HPLC

S.No.	Standard→ Genotypes↓	*p*-Hydroxy benzoic Acid (µg/ml)				Vanillic Acid (µg/ml)				Salicylic Acid (µg/ml)				Ferulic Acid (µg/ml)			
1.	HD2733	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		.016	.018	.021	.021	.051	.081	-	-	.085	.066	-	-	.020	.023	.024	.027
2.	PBW533	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		-	-	-	-	-	.336	-	-	-	.014	.025	.033	.001	.002	.010	.001
3.	DBW71	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		-	-	.013	-	-	-	-	-	-	.174	-	.079	.008	.099	.053	.118
4.	K9107	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		.010	.010	.013	.010	-	-	-	-	.078	.078	.283	.293	.011	.022	.023	.047
5.	PBW373	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		.009	.010	.016	.016	-	-	-	-	.139	.160	.175	.190	.050	.052	.121	.131
6.	K6525	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3
		-	-	-	-	-	-	-	-	.022	.024	.024	.026	-	-	-	-

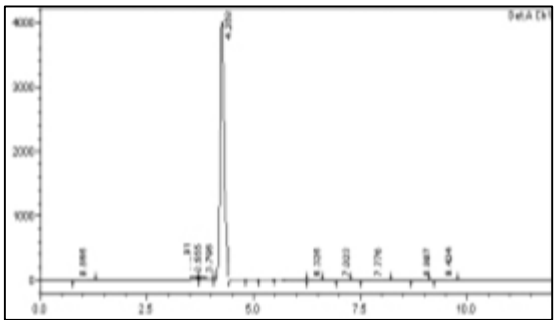
(Spot1-*p*-Coumaric acid, Spot2- Ferulic acid, Spot3- *o*-Coumaric acid, Spot4- Caffeic acid, Spot5-Sinapic acid)

(Spot1-Salicylic acid, Spot2- *p*-Hydroxybenzoic acid, Spot3- Vanillic acid)

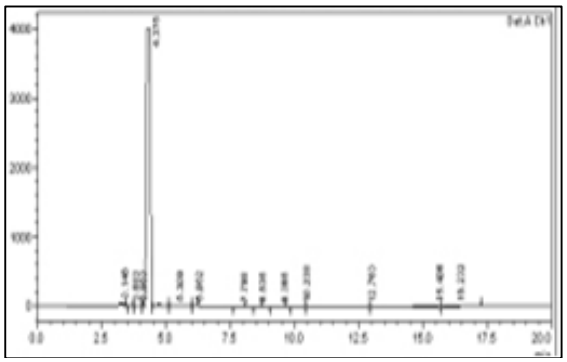
Fig 1: TLC Chromatograms showing different standard colour obtained in benzene:Acetic acid: water solvent system



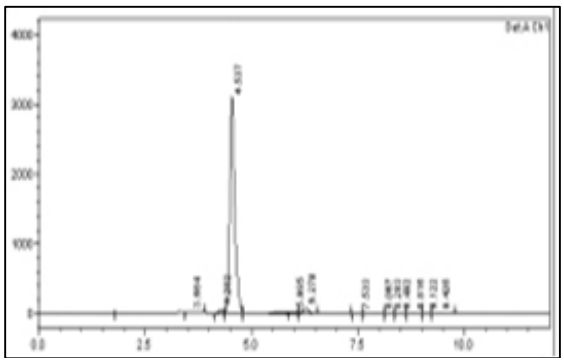
Caffeic (3.425)



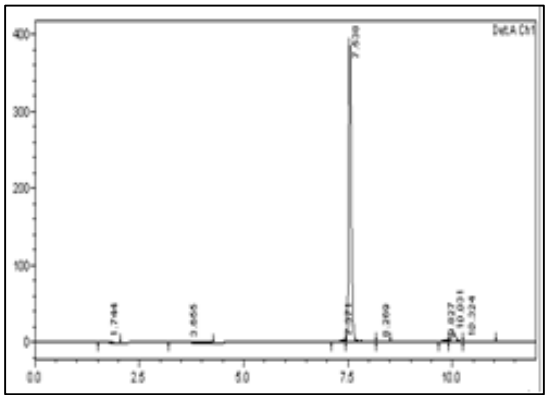
Sinapic (4.458)



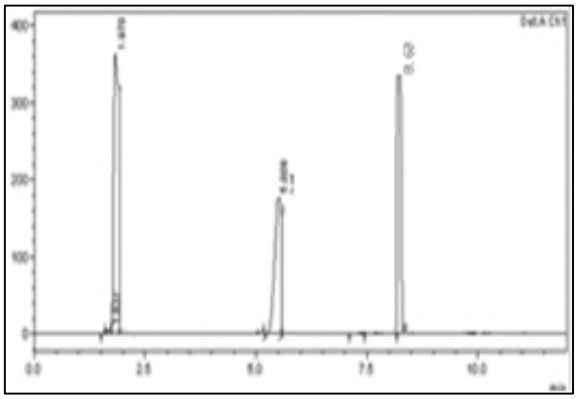
p-Coumaric (4.375)



o-Coumaric (4.537)



Ferulic (7.538)



p-HydroxybenzoicAcid (1.907), Vanillic Acid (5.245), Salicylic Acid (8.279)

Fig 2: Chromatographic separation of standard phenolic acid with their Retention time using HPLC

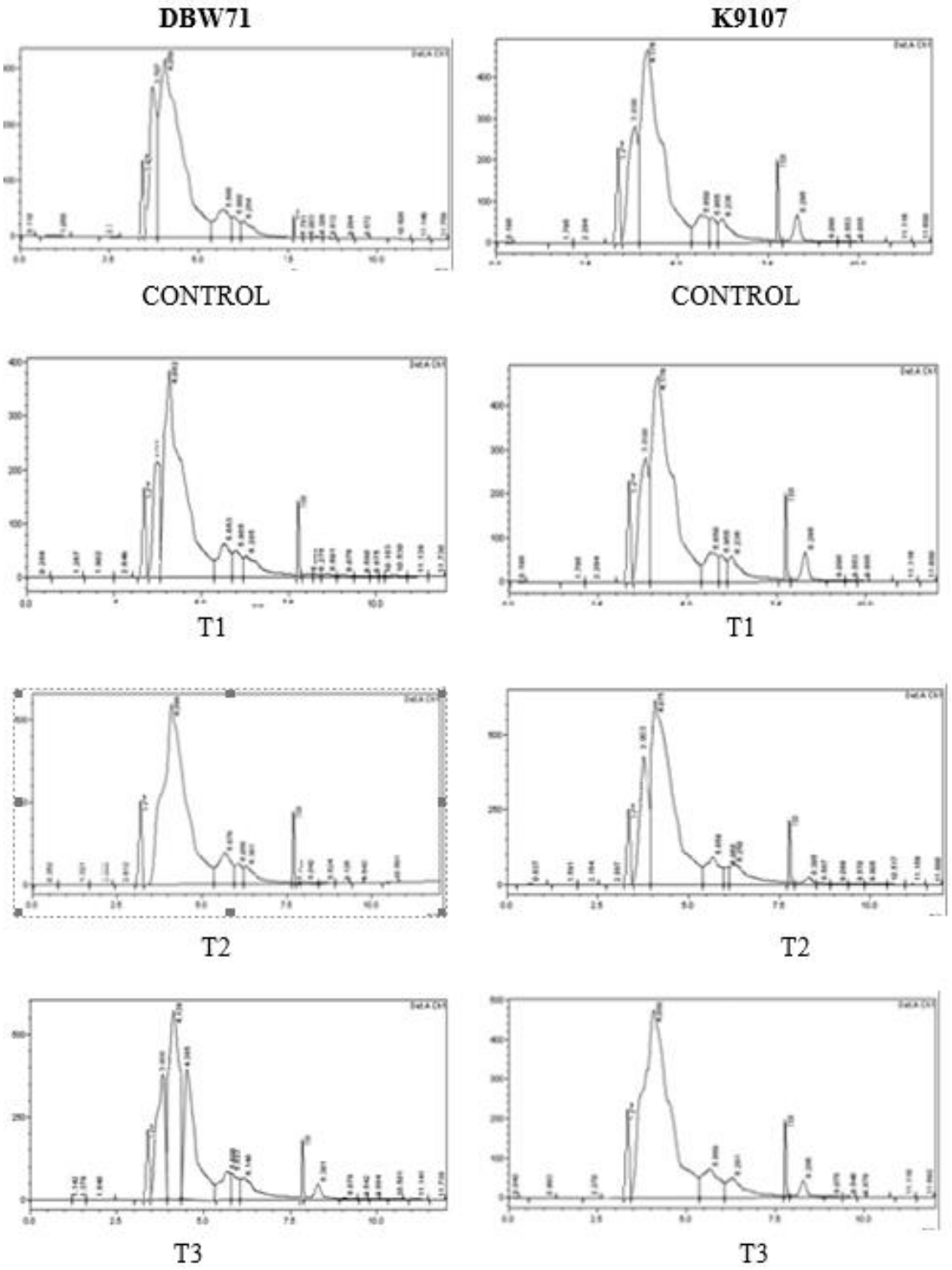


Fig 2 (B): HPLC Chromatograms of phenolic acids in genotype DBW71 and K9107 (Control, T1, T2, and T3)

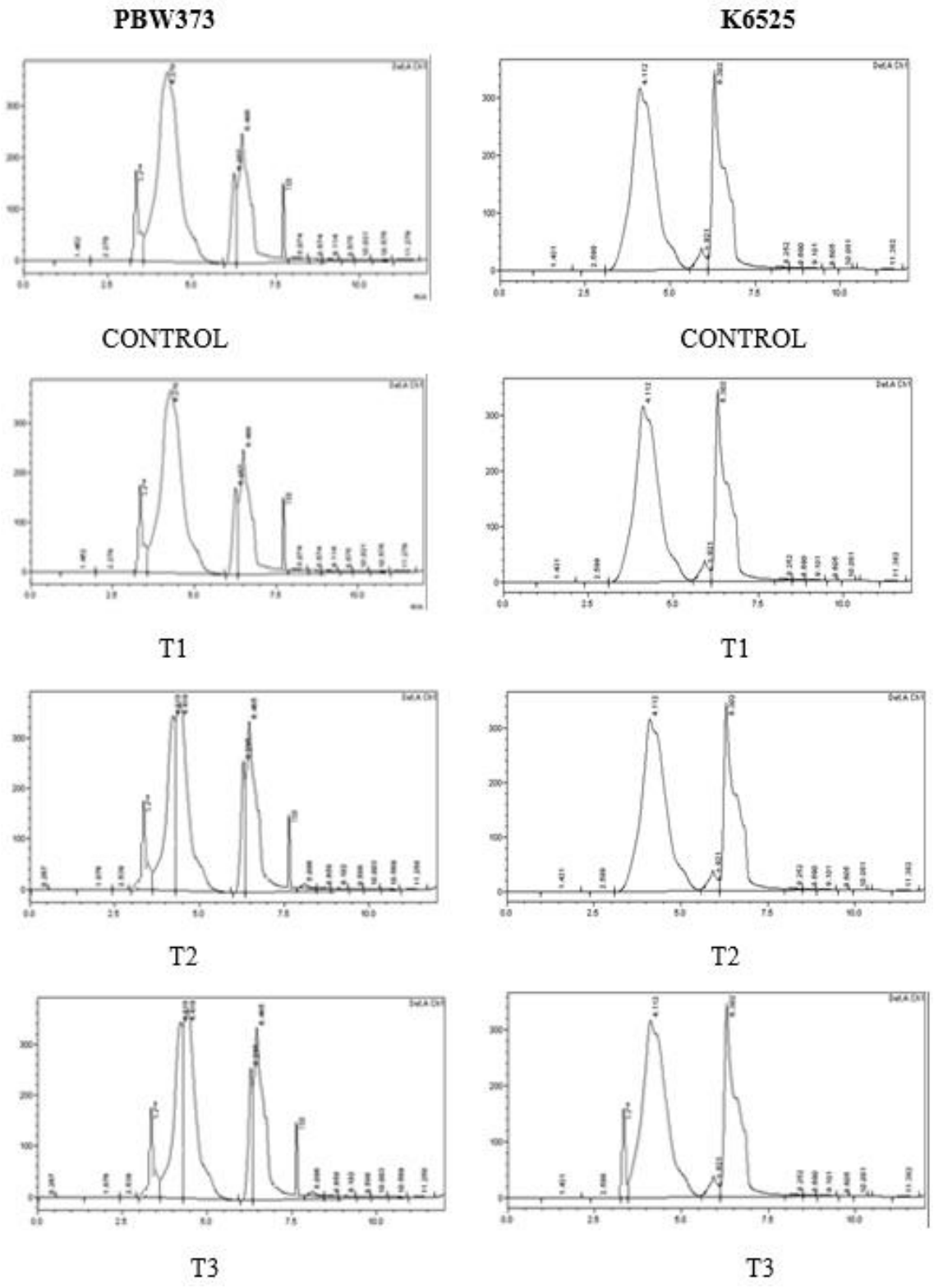


Fig 2 (C): HPLC Chromatograms of phenolic acids in genotype PBW373 and K6525 (Control, T1, T2 andT3)

Discussion

Plants often synthesize a series of chemicals with various bioactivities in response to specific stresses (Inderjit *et al.* 2006) [3]. Phenolic acids plays an important role in plants, undergoing stressed conditions. Their accumulation increases the stress tolerance ability of the plant. The ability to tolerate the drought stress of phenolic acids was reported to differ greatly among plant genotypes (Sabar and Arif 2014) [11]. In the present work, TLC and HPLC was performed, in which the TLC of different genotypes for the detecting the presence of p-coumaric, *o*-coumaric, caffeic, ferulic, sinapic acid, salicylic acid, p-hydroxybenzoic acid and vanillic acid in the leaves sample extract. The present study concluded with the detection of ferulic, caffeic, sinapic, p-hydroxybenzoic acid, salicylic acid and vanillic acid when subjected to TLC which was further confirmed through HPLC. ssHence, increased percentage of phenolic accumulation was recorded in leaves of wheat genotype. A study reported the detection of ferulic acid from wheat bran extract by inducing biotic stress using *Streptomyces* isolates (Sarangi *et al.* 2009) [12]. Some also reported the presence of p-hydroxybenzoic acid, vanillic acid, syringic acid, vanillin, ferulic acid, p-coumaric acid, benzoic acid and cinnamic acid in Rice under drought stress (Quan *et al.* 2016) [9]. Previous studies highlighted the accumulation of

phenolic acids and flavonoids as antioxidants and sunshields involved in responses of plants to drought stress and ultraviolet radiation (Nichols *et al.* 2015) [6]. The bioactivity of leaf phenolic molecules is considered as a signal trigger that leads to protective mechanisms against drought stress (Akula and Ravishankar 2011) [1]. Water stress resulted in synthesizing a large amount of flavonoids and phenolic acids in wheat leaves (Ma *et al.* 2014) [4].

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