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Quality RNA isolation, cDNA synthesis and qPCR validation of differentially expressed gene in *Punica granatum* L. under influence of *Xanthomonas axonopodis* pv. punicae

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Abstract
Bacterial blight is a widespread disease in pomegranate that causes great loss to farmers. The causative agent for this disease is *Xanthomonas axonopodis* pv. punicae. The present study was taken up to standardize protocol for quality total RNA extraction from various tissues of pomegranate, cDNA synthesis and qPCR validation of differentially expressed gene(s) identified from RNA sequencing data of susceptible and moderately resistant pomegranate genotypes upon challenge inoculation using qPCR. In the study, Phenol-Chloroform, Modified CTAB-LiCl and Trizol methods were evaluated for their efficiency to extract quality total RNA from infected and uninfected leaf and fruit tissues of pomegranate genotypes (Bhagwa and IC-1181). The concentration of extracted total RNA were quantified using Qubit Fluorometer and Qiagen QIAxpert and the quality of 18 and 28S bands of ribosomal RNA also assessed on agarose gel electrophoresis. Phenol-Chloroform method gave the highest concentration of total RNA having Qubit Fluorometer and QIAxpert readings ranged from 3.86 to 5.78 ng/µl and 543.5 to 1684.3 ng/µl, respectively. The time consumed and cost incurred on total RNA isolation were also least in Phenol-Chloroform method (50 minutes and Rs. 29.75/sample, respectively) as compared to other methods. From the high quantity total RNA, cDNA were synthesized using cDNA synthesis kit (HiMedia cDNA synthesis kit) and Xyloglucan endotransglucosylase coding gene was validated using qPCR. The qRT-PCR results showed that gene which code for Xyloglucan endo transglycosylase was slightly over expressed in the infected leaf samples of Bhagwa at infection stage 1 and 3 as compared with the control sample whereas the same gene had under expression in infected leaf sample of IC 1181 as compared to control.

Keywords: Pomegranate, total RNA, *Xanthomonas axonopodis* pv. punicae, qPCR

Introduction
Pomegranate (*Punica granatum* L.) is an ancient fruit tree from Iran. Its cultivation has remarkably increased by more than ten folds within a short span of about little more than 2 decades. Touched an all-time high of 0.226 million hectares in recent years with the production of approximately 2.6 million tons (http://www.nhb.gov.in). It is well adapted to drought and having distribution extending from Iran to Himalaya in Northern India and has been cultivated throughout the Mediterranean region [1, 2]. In recent years pomegranate has acquired central place in the fruit basket of the semi-arid regions of India because of its versatile adaptability, high market demand, more returns, less irrigation water requirement, excellent keeping quality, large array of high-quality value-added products, nutraceutical and pharmacological properties [3-6]. Pomegranate juice has been analyzed to have antioxidant capacity and it is good source of sugar, vitamin B, vitamin C and a fair source of iron. These specialized and peculiar features have contributed to the inclusion of pomegranate into the coveted category of 'Super Fruit'.

Bacterial blight is a widespread disease that causes damage to pomegranate fruit and the causative agent is *Xanthomonas axonopodis* pv. punicae [7]. This disease causes upto 80% of the total yield loss in pomegranate under serious epiphytotic conditions-a serious concern for farmers and pomegranate industry [8]. Bacterial blight was first time reported in India from Delhi [9]. Presently, the disease occurs widely and outbreaks has been reported in all major pomegranate growing state including Maharashtra, Karnataka and Andhra Pradesh. The disease has also been reported from South Africa [10], Pakistan [11] and recently from Turkey [12].

All the growth stages of plant is susceptible to blight and it only affects above ground plant parts, especially leaves, twigs and fruits. Disease buildup is rapid during monsoon. The most favorable condition for onset of blight as well as completion of disease cycle was when temperature ranged between 25-35 for at least 16 h and relative humidity> 30% for 24 h. [8].
Though, work on *Xap* related effector proteins, draft genome of *Xanthomonas axonopodis* pv. punicae , transcriptome analysis based simple sequence repeats (SSRs) and also single nucleotide polymorphisms (SNPs), transcript marker enriched genetic map, whole genome sequencing and tissue specific transcriptome analysis are well documented in pomegranate [13-18], but still host pathogen response with respect to *Xap* and pomegranate based on RNAsq data and its validation is not available in the public domain. Present study focused on challenge inoculation of leaf and fruit tissues of bacterial blight susceptible 'Bhagwa' and moderately resistant genotype 'IC-1181', quality total RNA extraction using different protocols, cDNA synthesis and qRT-PCR validation of differentially expressed genes obtained through RNA sequencing of challenge inoculated and control leaf and fruit tissues of bacterial blight susceptible 'Bhagwa'and moderately resistant genotype 'IC-1181'.

Materials and Methods

Bacterial strain and its multiplication

The pure culture of *Xanthomons axonopodis* pv. punicae maintained on Nutrient Glucose Agar (NGA) containing peptone, beef extract powder, dextrose, agar was inoculated in Nutrient Glucose Broth (NGB) and incubated on shaker at 100 rpm. After 48-72 h., growth of *Xap* in broth was observed; then broth was diluted with water and sprayed on both the genotypes of pomegranate.

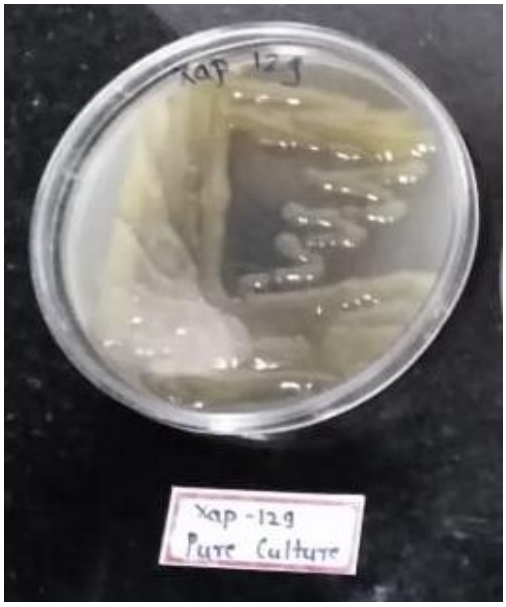


Fig 1: Pure culture of *Xap*

Plant material and challenge inoculation

About 8 to 10 months old air layered saplings of two pomegranate genotypes namely, 'Bhagwa'- the most commercial but highly susceptible to pomegranate bacterial blight and IC 1181- moderately resistant but wild type with small acidic fruits, were taken for challenge inoculation. The saplings were maintained in polyhouse of ICAR-NRCP in pots having pre sterilized potting mixture (sand: soil: FYM, 1:1:1) and prior to inoculation, the plants were subjected to high humidity for 24 h by covering them polythene bag and fastened with 1 ml pipette tip at the top for air exchange. The plants were then challenge inoculated with optimally diluted *Xap* broth by spray method twice at three days interval [8].

Symptom based and PCR based detection of *X. axonopodis* pv. punicae

Tissues developed symptoms after challenge inoculation were confirmed visually and through gyrB specific PCR based detection. The total genomic DNA of *X. axonopodis* pv. punicae were extracted from infected leaves with slight modifications [16].

Sample collection, total RNA extraction and cDNA synthesis

In Bhagwa there were three stages of infection; the leaf and fruit tissues having these different stages of infection were collected and leaf samples without infection were also collected. From IC-1181, infected leaf and fruit tissues along with control leaf and fruit samples were collected. Total RNA from infected and control leaf and fruit tissues of susceptible and moderately resistant pomegranate genotypes were extracted using three different methods of RNA extraction namely, Phenol chloroform, Modified CTAB LiCl method and Trizol method.

Modified CTAB-LiCl method

In modified CTAB-LiCl method about 100mg of finely crushed samples were transferred to 2ml centrifuge tube with 1ml of extraction buffer [2.5% CTAB, 100MmTris HCl (pH 8.0), 1.5M NaCl, 0.2% β-mercatothanol, 1% PVP]. The mixture was incubated at 60 for 30mins and centrifuged at 12,000rpm for 15 min at 4 °C. Then supernatant was collected in new centrifuge tube and equal volume of Chloroform: Isoamyl alcohol (49:1) was added. The above mixture was centrifuged at 12,000rpm for 15mins; supernatant was collected and equal volume of phenol chloroform added (1:1). Then mixture was centrifuged at 12,000rpm for 20 min and in supernatant 1ml 10M LiCl added and incubated at -20°. again 1ml 10M LiCl added in pellet after centrifugation at 12,000rpm for 20mins ; then 3M sodium acetate and ethanol is added for precipitation and incubated overnight at 4 °C. After incubation mixture is centrifuged at 12,000rpm for 15mins; then pellet is washed with 100% ethanol and pellet was dried at 37 °C and dissolved in nuclease free water.

Trizol method

The 100mg sample crushed in liquid nitrogen and transferred in 1ml of Trizol reagent and a pinch of PVP was added. Mixture is vortexed and incubated for 5min at room temperature; then centrifuged at 14,000rpm for 10 min and in supernatant equal volume chloroform was added and homogenized for 15 sec. The mixture centrifuged for 20min at 14,000rpm; aqueous phase was collected and 500µl of isopropanol was added and centrifuged at 14,000rpm for 10 min at 4 °C. Then pellet is washed with 75% chilled ethanol and air dried and dissolved in nuclease free water.

Phenol-chloroform method

100mg sample was crushed using liquid nitrogen and then 250µl ICAR-NRCP buffer and 250µl of phenol-chloroform was added and incubated for 15 min at 65 °C. Then mixture was centrifuged at 13000rpm for 5 min at 4 °C; in supernatant 500µl phenol-chloroform was added and centrifuged at 13000rpm for 2 min. In supernatant 50µl of 3M sodium

acetate and 500µl of 2-propanol was added and incubated on ice for 15 min and then centrifuged at 13000rpm for 10 min 4 °C. Then pellet is precipitated with 70% ethanol and then air dried and dissolved in nuclease free water.
The quality of extracted RNA was checked on gel electrophoresis and also by using Qubit Fluorometer and Qiagen QIAxpert.

cDNA synthesis
Total RNA was reverse transcribed into cDNA with primer and reverse transcriptase (HiMedia cDNA synthesis kit) in 15µl of total reaction volume as : 2µl RT buffer,0.8µl 100mM dNTP, 2.0µl primer, 1.0µl Multiscript RTase, 5µl of total RNA and 4.2µl nuclease free water. Then loaded it into thermal cycler; PCR cycles were programmed as: denaturing at 25 °C for 10 min,120 min annealing at 37 °C and 5 min elongation at 85 °C. cDNA was quantified using Qiagen QIAxpert.

Validation of differentially expressed gene by qPCR
The expression of the Xyloglucan endotransglyacosylase (primer sequence, forward primer: CCTCCCACAGGGTAGAGTAG, reverse primer: GAAAGGGTGACAGAGAACAGAG) was evaluated in leaf transcripts of 'Bhagwa' and 'IC-1181', qPCR. The qRT-PCR reactions were performed on Applied Biosystems StepOne Plus 96 well system using the Hi-SYBr Master Mix (HiMEDIA, India). Each reaction was performed 5 µlSYBR Green Master, 1 µl template cDNA, 0.2 µl each of the primers (10 µM), and 3.6 µl RNase-free water with a total volume of 10 µl. The qRT-PCR profile was as follows, 95 °C (2 min), 40 cycles of 95 °C (5 s), 60 °C (30 s) with fluorescent signal recording and 72 °C for 30s. The melting curve was obtained using a high-resolution melting profile performed after the last PCR cycle, 95 °C for 15 s followed by a constant increase in the temperature between 65 °C (15 s) and 95 °C (1 s).

Results and Discussion

Confirmation of *Xap* infection
Both morphological symptoms and molecular tool were used to confirm the *Xap* infection (Fig 2 and Fig 3). No amplification was observed in leaf control of IC 1181 and Bhagwa, the infected fruit and leaf samples produced amplicon size of 491 bp confirming *Xap* infection and the confirmatory results are in coherence with the results obtained by Mondal *et al.* [16] and Sharma *et al.*, 2017 [8].

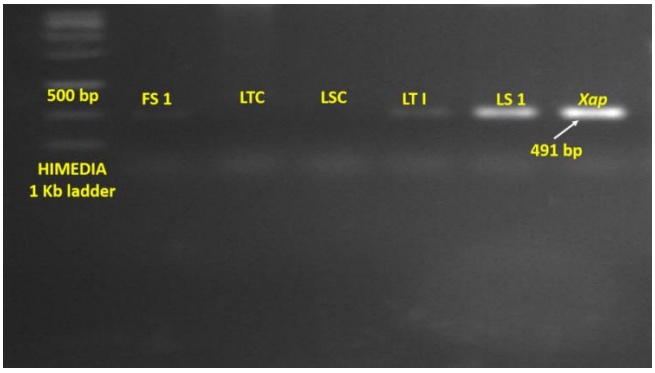


Fig 2: Confirmation of *Xap* infection using Gyrase B primers

First stage Second stage Third stage

Fig 3: Stages of infection observed on leaf of 'Bhagwa' plants

Quality and quality checking of extracted RNA
RNA was analyzed through gel electrophoresis; as per our result it was clear that RNA obtained from Phenol chloroform method was of high quality as compared to other two methods (Fig 4). Gel electrophoresis of total RNA showed two distinct 28S and 18S ribosomal bands without smearing, representing intact isolated RNA. RNA was quantified by using Qubit Fluorometer with RNA high sensitivity assay kit(concentration ranged from 3.86 to 5.78 ng/µl) and QIAxpert reading at 260nm (543.5 to 1684.3 ng/µl).Concentration of RNA extracted from leaf and fruit tissues using phenol-chloroform method were found to be more than modified CTAB-LiCl method and Trizol reagent method. (Table 1). The variable quality and quantity of extracted RNA may be due to high sensitivity of RNAs to various factors, contamination with protein and genomic DNA, presence of ribonucleases leading to RNA degradation and presence of high quality of polysaccharides, polyphenols and other secondary metabolites [19]. Earlier, Zarei *et al.* (2012) [20] and later Singh *et al.*, (2015) [2] tested various protocols of RNA extraction from different parts of pomegranate and found modified CTAB-based procedures better as compared to other methods but in our study we report Phenol-Chloroform based method as the best method for isolating quality total RNA at low cost and less time for various downstream applications.

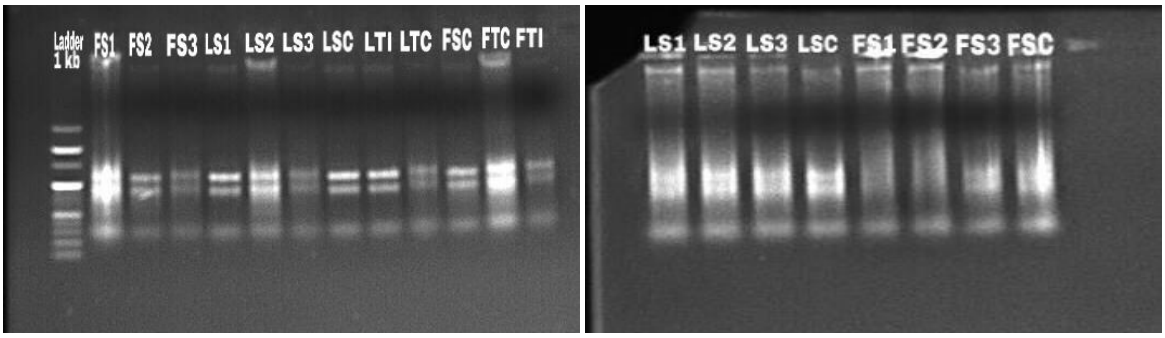
Table 1: Concentration of total RNA extracted by phenol- chloroform method using Qubit

Sample	Qubit reading using high sensitivity assay (ng/µl)	QIAxpert reading at 260nm (ng/µl)*
(LS1) Bhagwa leaf infected 1st stage	5.13	1684.3
(LS2) Bhagwa leaf infected 2nd stage	5.78	735.2
(LS3) Bhagwa leaf infected 3rd stage	4.89	1396.2
(LSC) Bhagwa leaf control	5.09	994.6
(FS1) Bhagwa fruit infected 1st stage	5.67	984.8
(FS2) Bhagwa fruit infected 2nd stage	3.86	543.5
(FS3) Bhagwa fruit infected 3rd stage	5.39	780.97
(FSC) Bhagwa fruit control	4.76	737.4
(LTI) IC-1181 leaf infected	4.88	878.3
(LTC) IC-1181 leaf control	3.17	168.5
(FTI) IC-1181 fruit infected	4.97	1259.5
(FTC) IC-1181 fruit control	5.23	1417.9

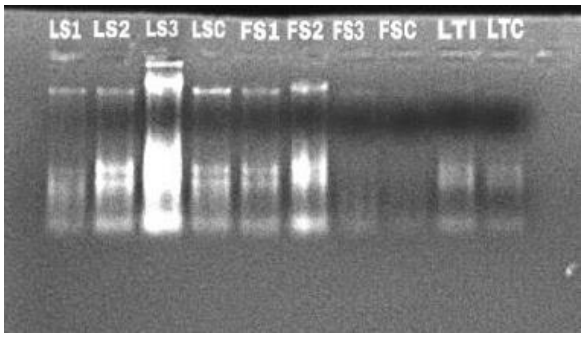
* Actual concentration may be very less due to high background and impurities

Table 2: RNA concentration Readings generated through Qiagen QIAxpert for samples extracted through Phenol-Chloroform method

Position	Sample Name	A260 Concentration (ng/ul)	Background (A260)	Absorbance at 260 nm
A1	blank_A1	0.0	0.00	0.00
B1	LS2	735.2	44.01	18.38
C1	LSC	994.6	42.25	24.87
D1	FS2	543.1	26.69	13.58
E1	FSC	737.4	46.39	18.43
F1	LTC	168.5	118.82	4.21
G1	FTC	1417.9	55.02	35.45
A2	LS1	1684.3	39.00	42.11
B2	LS3	1396.2	43.44	34.90
C2	FS1	984.8	24.26	24.62
D2	FS3	780.97	83.69	10.52
E2	LTI	878.3	50.28	21.96
F2	FTI	1259.5	53.57	31.49



Phenol- Chloroform method Modified CTAB- LiCl method



Trizol reagent method

Fig 4: Gel electrophoresis image of total RNA isolated from infected and control leaf and fruit peel tissue using three methods

Table 3: Time and Cost of different methods used

S. No.	Method	Time	*Cost per sample (Rs.)
1.	Phenol Chloroform Method	50 min.	29.75
2.	Modified CTAB-LiCl Method	72 h.	32.25
3.	TRIzol Method	2 h.	116.71

cDNA synthesis

From the high quality of RNA, cDNA was synthesized using HiMedia cDNA synthesis kit.

Table 4: cDNA concentration reading generated through Qiagen QIAxpert for two randomly selected samples

Position	Sample name	A260 conc. (ng/µl)	Background (260)	Absorbance at 260 nm
G2	Blank	0.00	0.00	0.00
H1	LS3	1500.1	31.50	45.46
H2	LS1	1810.6	22.13	54.87

qPCR validation

The validation of differentially expressed genes obtained from RNA seq data generated from different pomegranate tissues upon challenge inoculation with *Xap* was performed by using qPCR. The expression of gene coding for xyloglucan endo transglycosylase was validated. The gene was randomly selected from the data generated from DEGseq analysis. The forward and reverse primer was designed for the gene and gene was amplified in all the leaf samples of 'Bhagwa' and 'IC 1181' (Fig 5). The qRT-PCR results showed that gene which code for Xyloglucan endo transglycosylase was slightly over expressed in the infected leaf samples of Bhagwa at infection stage 1 and 3 as compared with the control sample. Similarly, when the infected leaf samples of IC 1181 is concerned, then we observe the under expression of the gene which code for Xyloglucan endo transglycosylase at first stage of infection than its control (Table 5). We found that the genes Xyloglucan endo transglycosylase was found to be differentially expressed and found in coherence with the DEGseq analysis (Fig 5). At the time of pathogen infection, generally carbon metabolism is high because of the upregulation of associated genes, which turns on the defense mechanisms by the activation of defense genes [21] and Xyloglucan endo transglycosylase is an essential component of signaling pathway involved in plant-pathogen interaction [22].

Table 5: Relative quantification data

Sample Name	Target Name	ΔΔCT	Relative quantification (2^-delta delta CT)
LS1	Target 1	-0.12761	1.09248
LS2	Target 1	1.781166	0.290948
LS3	Target 1	-0.22296	1.167126
LSC	Target 1	0	1
LT1	Target 1	-1.15097	2.150967
LTC	Target 1	-2.33239	5.036396

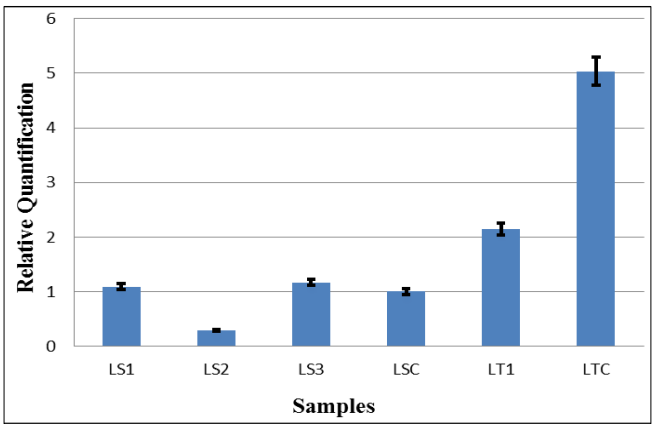


Fig 5: Relative quantification of Xyloglucan endo transglucosylase

Conclusion

The study confirms the effectively of Phenol-Chloroform method of total RNA isolation from different tissues of pomegranate for further downstream application. The qPCR validation of DEGseq analysis results for differential expression of xyloglucan endo transglycosylase in different pomegranate tissues upon challenge inoculation indicate the role of this gene in host pathogen interaction in *Xap*-pomegranate system.

Acknowledgements

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Conflicts of Interest

The authors declare that there are no conflicts of interest

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