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Effect of elevated temperature on quorum sensing signal molecule (N-Acyl Homoserine Lactone) production of *Pectobacterium carotovorum* subsp. *carotovorum* in tomato

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Abstract: Gram negative plant pathogenic bacteria regulate specific gene expression in a population density dependant manner by sensing level of Acyl-Homoserine Lactone (HSL) molecules which they produce and liberate to the environment, called Quorum Sensing (QS). The production of virulence factors (extracellular enzyme viz. cellulase, pectinase) in Pectobacterium carotovorum subsp carotovorum (Pcc) is under strong regulation of QS. The QS signal molecule, N-(3-oxohexanoyl)-L-Homoserine Lactone (OHHL) was found as the central regulatory system for the virulence factor production in Pcc and is also under strict regulation of external environmental temperature. Under seven different incubation temperatures (24° C, 26°C, 28°C, 30°C, 33°C, 35°C and 37°C) in laboratory condition, highest amount of OHHL (804 violacein unit) and 3-unsubstituted Hexanoyl Homoserine Lactone (HHL) and highest (79 %) Disease Severity Index (DSI) was measured at 33°C.

The OHHL production kinetics showed accumulation of highest concentration of OHHL at the exponential phase of the growth but diminution in the concentration occurred during stationary phase onwards to death phase. Instability of HSLs was increased at high temperature (35° C and 37°C) exposure and OHHL was not at detectable range. The effect of temperature on virulence factor production is the concomitant effect of HSL concentration which justifies less disease severity index in cross inoculated tomato fruits incubated at 35° and 37°c. The non-detection of the OHHL in the elevated temperature may because of degradation as these signal molecules which are quite sensitive and prone to get degraded under different physical factors like temperature.

This result provides the rationale behind the highest disease severity up to certain elevated temperature and leaves opportunities for investigation on mutation, co-evolution of superior plant pathogen with more stable HSL signals mediated pathogenesis under Global warming context.

Key words: Quorum sensing, Acyl-Homoserine Lactone (HSL), Elevated temperature, soft rot.

INTRODUCTION

Pectobacterium carotovorum pv. *carotovorum* causes soft-rot diseases in various commercially important crop plants including potato, tomato, carrot. Pathogenesis in *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *atrosepticum* (and other soft-rot *Erwiniae* sp) is dependent on production of an arsenal of plant cell wall-degrading enzymes that are actively secreted by the bacterium. The production of these exoenzymes which is the main virulence factor in soft-rot *Pectobacterium* species is governed by a cell density-dependent manner and such mechanism now known generically as quorum sensing [1]. Thus through quorum sensing, genes for virulence and pathogenicity in bacteria are subjected to regulation by sensing population density dependant transcriptional factors, post transcriptional mechanisms, indigenous signals, and signals of host origin, as well as environmental factors. One of the latter factors, temperature, has been found to play a critical role in numerous instances. For instance, virulence genes of *Yersinia* species are activated at 37°C [2].

Toxin production by several plant-pathogenic bacteria for example, production of phaseolotoxin by *Pseudomonas syringae* pv. *phaseolicola* and production of coronatine by *P. syringae* pv. *coronafasciens* is highly susceptible to high temperatures since little or no toxin production results from growth at 20°C or a higher temperature [3, 4, 5, 6]. In *Pectobacterium carotovorum* pv. *carotovorum*, apparent cell density-dependent exoenzyme production is regulated by a prior synthesis of an inducing concentration of the acyl HSL, *N*-3-(oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL).

As it is well established that QS is mandatory for a successful pathogenesis, and the current climate change trend is obvious, so in this context the pathogenic bacteria will definitely adapt in a most fitting way for successful cross talk and thereafter the induction of the specific target genes.

Keeping this view in mind current experiment was conducted with our isolated *Pectobacterium carotovorum* pv. *carotovorum* (IS1, Gene bank Accessio no. GU590785) strain to investigate the effect of different temperature conditions (which is normally encountered by changing climate during crop cultivation) on the kinetics of C6-OHHL production mediated pathogenesis and also to see whether the strain was producing the same or different kind of signals in all cases and their respective concentration in the growth media.

MATERIALS AND METHODS

Bacterial strains and media

In the current experiment, we used our own isolate *Pectobacterium carotovorum* subsp. *carotovorum* strain (Gene bank accession no. GU590785) isolated from soft rot infested tomato var. Pusa Ruby obtained from vegetable research farm of Indian Agricultural Research Institute (IARI), New Delhi, India. Other strains are derivatives of *Pectobacterium carotovorum* subsp. *carotovorum viz.* MS1 which was used as virulent and reference soft rot plant pathogenic strain. JBC1 is avirulent and derivative of MS1. For biodetection and quantification of AHLs, two different biosensor strains *viz. Agrobacterium tumefaciens* NTL4 (pZLR4) and *Chromobacterium violaceum* (CV026) were used.

The AB minimal glucose–yeast medium used for culturing the *Agrobacterium* indicator strain contained: 1 g, yeast extract; 3 g, K_2HPO_4; 1 g, NaH_2PO4; 1 g, NH_4Cl; 0.30 g, $MgSO_4.\ 2H_2O$; 0.15 g, KCl; 10 mg, $CaCl_2.\ 2H_2O$; 2.5 mg, $FeSO_4.\ 7H_2O$ in 1 liter of distilled water, pH adjusted to 7.4 and autoclaved at 121^{o}C for 20 minutes. Filter-sterilized glucose solution (1 ml of 20% w/v solution per liter) was added to the medium before use. The CV026 was maintained in Luria Bertani Hiveg^{TM} (LB) medium but for extraction of the violacein pigment Tryptone -Yeast Extract (TY) medium was used [7]. For maintenance of the isolated *Pcc* strain, LB medium (Himedia, India) was used throughout the study.

Acyl-HSL Standards

Both L-HHL and L-OHHL was purchased from the Sigma Chemical Co. (St. Louis, Mo.) and was stored in -80^{o}C till further use.

Growth curves and culture conditions

A preculture of the IS1 strain was prepared by inoculation of IS1 in LB broth and growing at 28^{o}C for 16 hour at 160 rpm with around 1.2 O.D at 600 nm. 0.1 ml from this preculture of $2x10^7$cfu/ml was inoculated to 500ml of LB broth and then each conical was incubated at seven different temperature conditions viz. 24^o, 26^o, 28^o, 30^o, 33^o, 35^o and 37^o C with 160 rpm till 45 hours in each case. Samples were withdrawn in five different phase *viz.* initial log phase, mid log phase, late exponential phase, stationary and death phase for Optical Density, pH and to measure the OHHL concentration simultaneously.

Autoinducer (Quorum sensing signal) assay

For autoinducer assay the isolated strain was inoculated to 500 ml of sterilized LB broth in 1 liter conical flask. After inoculation the flask was incubated at 28^{o}C (when autoinducers are studied under optimum conditions) and was shaken in 160 rpm. To see different temperature and other physical environmental parameters on the autoinducer production, after inoculation was incubated at those conditions. This was performed in two separate conical flask with same amount of broth under same conditions. The total amount from the two flask was pooled into a single container. Reported that autoinducer is produced in highest amount in the near stationary or late log phase [8], for detection of signal molecules we extracted the culture at the late log phase.

Extraction of HSLs from IS1 culture supernatant

Extraction of HSLs was performed by following protocol given by Eberhard *et al.* [9]. For extraction and purification of the autoinducer we harvested the inoculated broth in the late log phase which was determined during growth curve assessment under same growth conditions. The cells were removed by centrifuging at 15,000 rpm for 10 minutes in 4^{o}C. Purification of autoinducer was performed by following protocol of Everhard *et al.* [9] with slight modification. Again the supernatant was extracted thrice by shaking for 5 min with 500 ml of redistilled ethyl acetate containing 0.1 ml/ L glacial acetic acid each time.

The combined ethyl acetate extracts were dried briefly over Na_2SO_4 and evaporated to dryness in vacuo by using rotary evaporator at 40^{o}C. The solid yellow oily material was extracted twice with 2 ml each of ethyl acetate. The total extracts were combined and evaporated to dryness. The resulting material was treated with 1 ml of Ethanol (95 %). Then 10 ml of water was added, which gave a cloudy suspension. This was then centrifuged for 10 min at 15,000 rpm and filtered through 0.2µ gelman filter. The filtrate gave a clear solution which was evaporated to dryness. Resulting yellow oily material was again extracted with ethyl acetate and the solution applied (60-200) mesh silica gel preparative TLC plate. The plate was run with a solvent system containing 90% ethyl acetate and 10% Methanol. By determining the R_f value from the standards, the active fractions were scraped out from the TLC plate and collected in separate vials.

The scrapped silica gels were extracted with 2 ml of ethyl acetate and the upper layer was separated carefully in separate vials and evaporated to dryness. The final white powdery material is 99% pure natural autoinducer.

Bioreporter strain mediated detection of HSLs through C18 RP-TLC

Agrobacterium tumefacience (NTL4) mediated detection of HSL compounds were performed by following protocol given by Shaw *et al.* [10].

Preparative TLC for partial purification of HSLs

From crude extracts of HSLs, C6-OHHL was purified by using preparative TLC this concentrated and purified OHHL was used for the OHHL kinetics study.

Quantification of HSL

C. violaceum (CV026) mediated quantification of the OHHL was performed following the protocol given by Blosser *et al*. [7].

RESULT

Detection of multiple AHLs produced by IS1 in the late exponential phase grown under different temperature exposure

Our isolated strain (IS1) produced multiple AHLs while grown under seven different temperature conditions. Biodetector strain *Agrobacterium* NTL4 mediated study unveiled three different types of HSL spots on the RPTLC plate. Among the seven different temperature treatment we could detect only C6-OHHL, C6-HHL in 24^o, 26^o, 30^o and 33^o C. But exceptionally and interestingly at 28^oC we could detect another novel compound C10-undecanoyl HSL along with C6-OHHL and C6-HHLwas not detected in 28^oC. The extracts from higher temperature treatments viz. 35^o, 37^o C neither yielded the C6-OHHL spot nor the C-10 HSL but only a weak characteristic spot of C6-HHL was detected (Fig.1 and Table. 2). In no case we could detect the C4-HSL (BHL). For confirmation of this exceptional result from 28^oC we have performed the Mass Spectrometric Analysis for identification of the novel spot with characteristic 0.19 Rf value and which gave 268 characteristic mass peak (MH^+) and was identified tentatively as C10- Undecanoyl HSL (Fig. 2).

Quantification of different levels of OHHL produced by IS1 in late exponential phase under different temperature conditions

AHL, the quorum-sensing signal, is required for exoenzyme production and virulence in *Pectobacterium* subspecies [11-13]. Among the AHLs produced by *Pectobacterium* sp, C6-OHHL played the central role in pathogenesis. As several reports showed a trend of highest HSL accumulation in the late exponential phase [8], in the current study we tried to investigate the impact of temperature on the central regulator for pathogenesis in *Pectobacterium* i.e. OHHL production and their accumulation in the near stationary or late exponential phase. Extracted and purified C6-OHHL from the late exponential phase spent culture strongly confirm the impact of the physical environmental factors like temperature on types and quantity of HSLs production by *Pectobacterium carotovorum* pv. *carotovorum* (IS1). The highest OHHL activity was noticed in 33^o C which was in the level of 804 violacein unit. In 24^o, 26^o, 28^o and 30^oC the activity of violacein unit was calculated as 288.07, 428, 453, and 585 respectively. From this result the impact of different temperature on the OHHL production in case of *Pectobacterium carotovorum* pv. *carotovorum* was crystal clear. In the elevated temperature at 35^o and 37^oC OHHL was not at the detectable level in the late exponential phase (Fig. 3). Elevated temperature exposure enhanced C6-OHHL production and accumulation in the late exponential phase. But this kind of trend was found till 33^oC. Further increase in the temperature viz. 35 and 37^oC showed a negative impact on the OHHL production. Infect, in this two temperature treatments in late exponential phase the OHHL level was below the detection level by CV026. When the same phase extraxct of 35 and 37^oC was spotted on the RPTLC plate for *Agrobacterium tumefaciens* (NTL4) mediated detection, we could not detect the OHHL which also supports our previous result of CV026 mediated failure of quantification of OHHL.

Kinetics of OHHL production under different temperature treatments

To investigate the impacts of temperature on the kinetics of OHHL production samples were withdrawn at five different growth phase viz. Initial log phase, mid log phase, late exponential phase, stationary phase, death phase and samples in each phase were processed for extraction, purification and quantification of OHHL. However, as there is difference in the generation time due to temperature, in all seven different temperature treatments, from the growth curve study the different phases were identified previously. So in all treatments only growth phase was considered rather sampling time interval as it may vary under different temperature exposures. Effect of different temperature viz. 24^o, 26^o, 28^o, 30^o, 33^o and 35^oC on the kinetics of OHHL production is shown in Fig. 4. In all the five temperature conditions, initially the OHHL concentration rose during the mid log phase of the growth curve and increased linearly (R^2 = 0.99) with the population cell density. However, as the cells entered the stationary phase, the OHHL concentration (in terms of Violacein units) reached a maximum value and then decreased as rapidly as it had accumulated (Fig. 4). However depending on the incubation temperature exposure, in the lower temperature treatments the initiation of the OHHL production took comparatively longer time than the higher temperature treatments because in the lower temperature the bacteria take longer generation or doubling time. At 24 ^{o}C and 26^oC a progressive increase in the OHHL production noticed till late exponential phases in which OHHL activity showed 288 and 428 violacein units. Only two degree temperature difference showed an almost double concentration of OHHL in terms of violacein units though the kinetics of OHHL production trend was similar. $(27\text{-}28)^o$ C, which is reportedly the optimum

temperature for *Pectobacterium carotovorum* pv. *carotovorum*, kinetics of OHHL production trend was similar with 24°C and 26°C but the initiation of the OHHL production started earlier (4 hr after inoculation) than the two previously mentioned temperature exposures. But in the late exponential phase of 28°C, the finally accumulated OHHL showed 543 violacein units. Though the two degree difference in case of 28°C did not show a marked difference in terms of OHHL kinetics pattern but in late exponential phase the cumulative concentration of the OHHL in 24, 26 and 28°C, was statistically different. The elevated incubation temperatures *viz.* 30°, 33°C elucidate a marked difference in terms of initiation of OHHL production and its final accumulation level in the late exponential phase. Among the seven different temperature treatments, 33°C exposure showed a maximum production and accumulation of OHHL (804 violacein unit) in the late exponential phase (Fig. 4). In both the cases of 30 and 33°C, detectable OHHL production started 3 hrs after inoculation. However, in the further elevated incubation temperature viz. 35 and 37°C exposure, the OHHL level was below detection limit of CV026 (Fig. 3).

Effect of elevated incubation temperature on the pathogenesis

Inoculation of tomato with *Pectobacterium* and thereafter incubation at previously mentioned seven temperatures showed maximum disease severity (79%) in 33°C incubation temperature. In the further elevated temperature viz. 35 and 37°C though the disease was started but, severity was found comparatively quite less than the earlier treatments (Fig. 5).

DISCUSSION

As previous reports confirmed about the instability and proneness to degradation of the signals under different physical factors of the surrounding environment viz. temperature, pH etc, current investigation was performed to see whether our isolated strain *Pectobacetrium carotovorum* pv. *cartovorum* (IS1) which is a soft rot causing plant pathogenic bacteria is really behaving similarly or differently in comparison to the optimum conditions. *Agrobacterium* (NTL4) mediated detection of AHLs produced by IS1 in the late exponential phase under different incubation temperatures, showed production of multiple AHLs of which the 3-oxo substituted OHHL and 3-unsubstituted HHL derivatives migrated with a characteristic Rf value along with characteristic spot shape. In the result of RPTLC, we also found OHHL and HHL with relative migration factors of 0.68 and 0.47 which is similar with Shaw *et al.* [10]. In addition, the 3-oxo derivatives (OHHL) characteristically produced tailing spots with diffuse edges, whereas the 3-unsubstituted forms (HHL) produced circular spots with sharp edges (Fig. 1) which previously also reported by Shaw *et al.* [10]. *Pectobacterium carotovorum* pv. *carotovorum* reportedly produce BHL, HHL, OHHL [8]. In contrast with this report we could detect another QS active compound near the base line of RP-TLC with a relative migration factor of 0.19 which was further confirmed by mass study as C10-Decanoyl Homoserine lactone with a MH^+ peak of 268. Previously Nasser *et al.* [14] also reported C10-HSL from *Pectobacterium carotovorum* pv. *crysenthemi*. However, in contrary with Byers *et al.* [8], in no case we could detect the BHL. Because this is minor HSL compound and relative abundance of it in comparison with the OHHL is very less, the BHL might have produced by our strain IS1 also, but which may was below detection limit (detection limit 40 nM). Moreover, the reason of nondetection of OHHL in the higher temperature exposure i.e. 35 °C and 37°C may be due to simultaneous production and degradation of the OHHLs during the growth in the medium. In a earlier report by Hasegawa *et al*. [15], *Pectobacterium carotovorum* pv. *atroseptica*, *Pectobacterium carotovorum* pv. *betavasculorum*, and *Pectobacterium carotovorum* pv. *carotovorum* produce high levels of extracellular enzymes, such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt) and the quorum-sensing signal *N*-acyl-homoserine lactone (AHL) at 28°C. However, the production of these enzymes and AHL by these bacteria is severely inhibited during growth at elevated temperatures (31.2°C for *Pectobacterium carotovorum* subsp. *atroseptica* and 34.5°C for *Pectobacterium carotovorum* pv. *betavasculorum* and most *Pectobacterium carotovorum* pv. *carotovorum* strains). At elevated temperatures these bacteria produce high levels of RsmA, an RNA binding protein that promotes RNA decay. *Pectobacterium carotovorum pv. carotovorum* strain EC153 is an exception in that it produces higher levels of Pel, Peh, Cel, and Prt at 34.5°C than at 28°C. EC153 also causes extensive maceration of celery petioles and Chinese cabbage leaves at 34.5°C, which correlates with a higher growth rate and higher levels of rRNA and AHL. The lack of pectinase production by *Pectobacterium carotovorum* subsp. *carotovorum* strain Ecc71 at 34.5°C limits the growth of this organism in plant tissues and consequently impairs its ability to cause tissue maceration. We found the similar result of Hasegawa et al. and could not detect the OHHL in the higher temperature treatment may be due to the level of OHHL produced in the higher temperature was not sufficient for *Agrobacterium* mediated biodetection or the OHHL produced but got degraded due to higher temperature exposure. In support to this discussion there is another chance of nonenzymatic turn over of the OHHL while growing in the medium.

The kinetics of OHHL production in all temperature treatments showed a similar trend of highest accumulation in the late exponential phase with an average population density of $3x109^{-11}$ cfu/ml and thereafter rapid decline in the OHHL concentration in the spent medium. This result confirms Byers et al. [8] report, where similar kind of trend was reported. This kind of kinetics trend of the OHHL by *Pectobactrium carotovorum* pv. *carotovorum* in the spent medium is dependant on the pH, temperature, oxygen and types of carbon sources in the media. Interestingly the growth media

pH was initially 7 but till death phase the pH started increasing and reached 8.7 in the death phase. Byers *et al.* [8] also reported that degradation of OHHL in the stationary phase was dependant on the pH of the supernatant which increased as the growth curve progressed in the cultures grown in the LB medium from 7 to ≈8.5 became unstable over a narrow pH range (pH 7 to 8). Instability was increased at high temperatures even at neutral pH but could be prevented at the growth temperature (30°C) by buffering the samples at pH 6.8. These results may provide a rationale for the observation that an early response of plants which are under attack by *Pectobacterum sp* is to activate a proton pump which alkalizes the site of infection to a pH of >8.2. We also found similar kind of alkalization of the LB medium along with the progression of the growth curve in all seven temerature exposures

CONCLUSION

From the current investigation, it can be concluded that production of the QS signal molecules is tightly regulated by surrounding temperature. More than 33oC showed degradation of the C6-OHHL and hence interrupted the QS mediated pathogenesis in the elevated temperature.

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Fig. 1: *Agrobacterium tumefaciens* (NTL4) mediated detection of AHLs produced by *Pectobacterium carotovorum* pv. *carotovorum* (IS1) under different temperature exposure

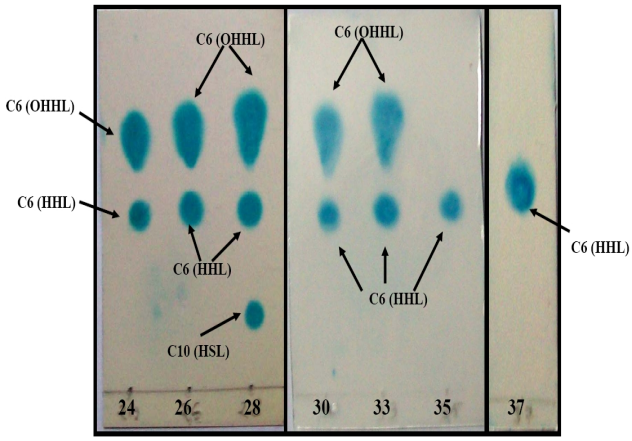

Fig 2: Mass spectra of quorum sensing signal molecules of *Pectobacterium carotovorum* pv *carotovorum* (IS1)

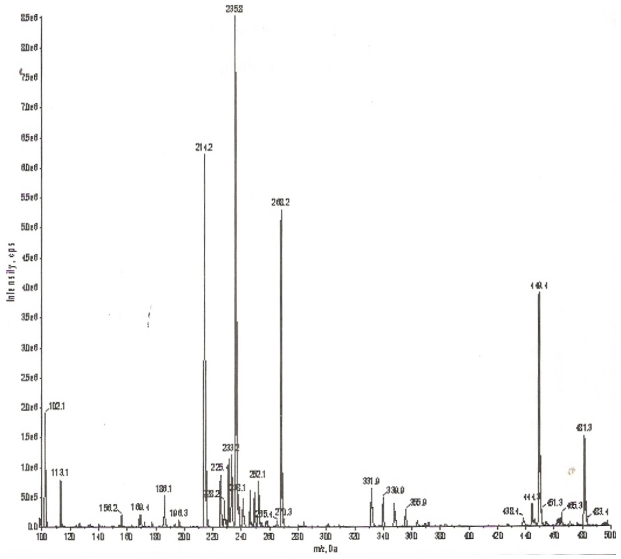

Fig. 3: Concentration of C6-OHHL in the late exponential phase extract of IS1 grown under different temperature treatments *viz.* 24°, 26°, 28°, 30°, 33°, 35°, 37°C

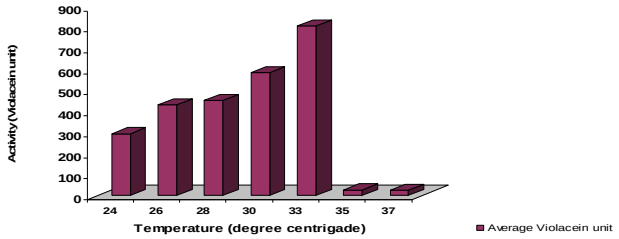

Fig. 4: Effect of different incubation temperatures on C6-OHHL production kinetics by IS1

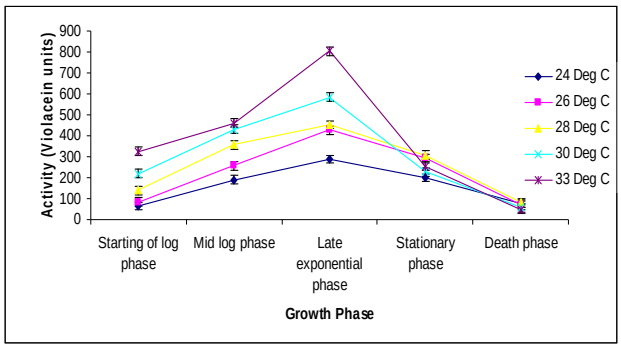

Fig. 5 Disease severity by *Pectobacterium carotovorum* pv. *carotovorum* under seven different incubation temperature in laboratory

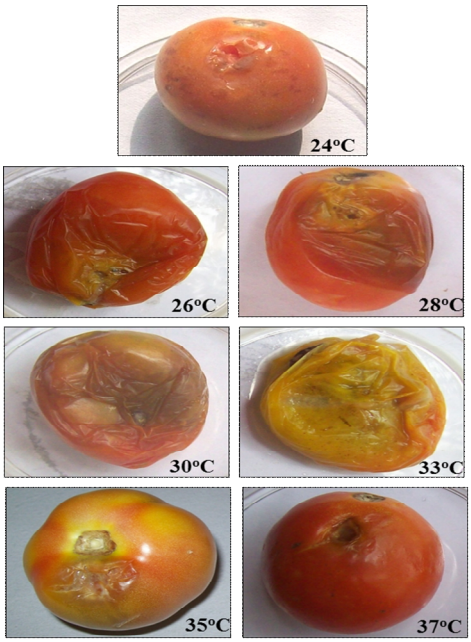

Table 1. *Agrobacterium tumefaciens* (NTL4) mediated detection of multiple HSLs produced by IS1 in the in the near stationary or late exponential phase under different temperature treatments

Treatment (°C)	24	26	28	30	33	35	37
C6-OHHL	+	+	+	+	+	-	-
C6-HHL	+	+	-	+	+	+	+
C10-HSL	-	-	+	-	-	-	-