Summary and Conclusions
5.1 Background and Objective: *Pseudomonas syringae* pv. *syringae* 61 *hrpZ* encodes harpin$_{Pss}$, a 34.7kD extracellular protein that elicits hypersensitive response (HR) in plants. We showed that conditional expression of harpin$_{Pss}$ causes yeast cell death hypothesizing that yeast might share, with plants, conserved components in cell death pathway (Podile *et al.*, 2001). We have also reported that harpin-induced plant HR and YCD are similar because: conditional expression of harpin$_{Pss}$ causes YCD, oxidative burst plays a role in harpin$_{Pss}$-mediated YCD and a protein kinase inhibitor (K252a) that suppresses plant HR also suppresses YCD.

With the above background, the present study was focused on: a) characterization of the conditional expression of harpin$_{Pss}$-mediated YCD and b) the structure-function relationship of harpin$_{Pss}$, an unusual peptide, causing cell death in both plants and *Saccharomyces cerevisiae* Y187.

5.2 Characterization of conditional expression of harpin$_{Pss}$-mediated YCD: Harpin$_{Pss}$ was expressed in *E. coli*, purified, polyclonal antibodies were raised in rabbit and also the protein was sequenced. Amino-terminal sequencing of the purified harpin$_{Pss}$ confirmed the start codon of harpin$_{Pss}$ and revealed the sequence similarity with the deduced amino acid sequence for the gene sequence available in the database.

A 1.02kb full-length *hrpZ* from *P.s. pv. syringae* was cloned into pYEUT under the control of the GAL1 promoter for conditional expression of harpin and cells were shifted to galactose-containing media from glucose-containing media. Plating of pYEUT-*hrpZ* transformants on semisolid medium containing galactose resulted in complete inhibition of colony formation, whereas growth on the glucose-based medium was unaffected. The western blot analysis confirmed the expression of harpin$_{Pss}$ in yeast cells expressing the *hrpZ* gene in pYEUT-*hrpZ* under the GAL1 promoter in galactose-containing medium within 1 h of induction. A time-dependent decline in the percentage of trypan blue-excluding cells in cultures of pYEUT-*hrpZ* transformants when cultured on galactose-containing medium was observed. In contrast, cells grown in glucose-containing medium remained mostly dye-negative. The number of colonies markedly reduced to about 50% within 6 h. By 24 h, very few viable colony-forming cells remained in the cultures. Thus, conditional expression of *hrpZ* resulted in irreversible inhibition of colony formation, consistent with a lethal phenotype.

Extracellular effect of harpin when studied, by adding different concentrations of harpins extracellularly ranging from 5μM to 20μM on pYEUT-*hrpZ* transformant of *S.*
cerevisiae Y187 cultured in glucose-containing medium, revealed that harpins had no effect on yeast cells cultured both in liquid and semisolid media and thus cells were growing normally. Extracellular expression of hrpZ, with the help of an alpha factor leader sequence also confirmed that harpin does not cause YCD from outside the yeast cells.

S- and M-phase arrested yeast cells after 3 h of induction indicated that harpinPss-mediated cell death occurred in both S- and M-phases, independent of the stage of cell cycle.

To assess chromatin condensation and nuclear morphology of the cells expressing harpinPss, DAPI staining, genomic DNA and EM analyses were carried out. DAPI stained cells grown in the presence of galactose did not show chromosomal condensation, which reveals that chromosomal DNA fragmentation does not seem to occur in the harpinPss-induced YCD. Analysis of genomic DNA on a conventional agarose gel electrophoresis confirmed that there was no genomic DNA fragmentation or ladder formation in cells cultured in galactose-containing medium suggesting that this feature of apoptosis was not seen in harpinPss-mediated cell death, while there was genomic DNA fragmentation, a typical marker of apoptosis in H$_2$O$_2$-treated cells. Electron microscopic observations further confirmed that the yeast cells expressing hrpZ revealed no evidence of chromatin condensation. Overall this study confirmed that there was no chromatin condensation and nuclear fragmentation in harpinPss-mediated YCD.

Possible loss of membrane integrity in harpinPss-mediated YCD when studied by staining the cells with FDA and PI simultaneously, cells cultured in galactose-containing medium fluoresced orange/red taking up PI, implying loss of membrane integrity, whereas the cells grown in presence of glucose were green, taking up FDA, implying that they had intact membrane, indicating that there could be loss of membrane integrity in harpinPss-mediated YCD.

Mitochondria play a central role in programmed cell death. To study the role of mitochondria in harpinPss-mediated YCD, 'petite' mutants (respiratory deficient mutant) of S. cerevisiae Y187 were generated by "margin of growth" technique and transformed with pYEUT-hrpZ. The transformants of 'petite' mutants of S. cerevisiae being insensitive to harpinPss-mediated cell death suggested the role of mitochondria in this form of YCD.

One of the major apoptotic pathways is accompanied by the release of cytochrome C (Cyt C) from mitochondria into the cytosol. Western blot analysis of the
cytosolic fractions of pYEUT-hrpZ transformant of *S. cerevisiae* Y187 cells cultured in galactose-containing medium revealed that there was no evidence for the leakage of Cyt C from the mitochondria into the cytosol. Cyclosporine A, a potent inhibitor of permeability transition pore formation, did not have an affect on harpin_{Pss}-mediated YCD.

The observed cell death in *S. cerevisiae* Y187 when studied in other strains of yeasts viz., *S. cerevisiae* DY150, W303, Sey6211, BY4741 and BJ2168, YCD was observed in pYEUT-hrpZ transformants of Sey6211 and BJ2168 similar to Y187, whereas, the other three strains were insensitive to harpin_{Pss}-mediated YCD.

A comparative account of described characteristics of plant HR and harpin-induced YCD is presented as Table 7.

5.3 **Structure-function relationship of harpin:** Harpin_{Psp} and harpin_{Ps} belong to the same family of proteins produced by *P. syringae*. Conditional expression of harpin_{Psp} and harpin_{Ps} under galactose-inducible promoter caused cell death of *S. cerevisiae* Y187 similar to harpin_{Pss}.

To study the effect of N-terminal and C-terminal deletions on cell death activity of harpin_{Pss}, twelve different mutants were generated by PCR-based approach by truncating either at N- or C-terminal end or either ends of the full-length harpin_{Pss}. When these mutants were cloned and expressed in *S. cerevisiae* Y187, all the mutants retained the biological activity, similar to the full-length *hrpZ* in terms of YCD.

An attempt was made to study the structure-function relationship of harpin using bioinformatic approaches. Homology existing between harpins when studied using Blast P program revealed that harpins are distantly related and there is less sequence similarity existing between them. So, harpin was blasted against Swissprot using PSI-BLAST and then homology was detected between the harpins of *Erwinia amylovora*, *E. chrysanthemi* and *E. carotovora*. A multiple sequence alignment (MSA) of these homologues revealed well-conserved individual amino acids and extended regions of high similarity, mostly at the C-terminal end. MSA of harpins from *Erwinia* sp. and *Pseudomonas syringae* showed very less similarity between them. Most of the conserved residues were present at the C-terminal end and notable feature observed in performing the MSA was, most of the glycines are conserved in harpins.

When harpin secondary structure was predicted using PHD algorithm, the results showed that all harpins have tendency to form alpha helices. *There is a report showing that Type III chaperone is known to have helical secondary structure. Attempt to predict the three-dimensional model of HrpZ of *P. syringae* was not successful since no significant homologues were found in the PDB databank.*
Table 7  Comparison of the characteristic features of plant HR and YCD.

<table>
<thead>
<tr>
<th>Features of HR/YCD</th>
<th>Plant HR</th>
<th>YCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triggered by interaction of ‘R-avr’ gene products</td>
<td>Yes</td>
<td>Not known</td>
</tr>
<tr>
<td>Triggered by pathogen-derived molecules like harpins</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Reduction in cell size and localized cell death</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Truncated harpins cause cell death similar to full-length <em>hrpZ</em></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Involves a serial signal transduction and <em>de novo</em> synthesis of transcripts and proteins</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Triggers oxidative burst</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Kinase activity blocked by K252a, a protein kinase inhibitor</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Features of apoptosis like membrane blebbing, formation of apoptotic bodies, etc.,</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Maintenance of plasmalemmal integrity</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Chromatic condensation and DNA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Involvement of mitochondria</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Leakage of cytochrome C from mitochondria into the cytosol</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
Since bio-informatic approaches to predict the structure of the harpin were unsuccessful, an attempt was made to crystallize harpin$_{Pss}$. Out of the different combinations used, 60% PEG and Tris.Cl pH 8.5 was ideal to create the supersaturated state for successful growth of harpin crystals.

5.4 MAJOR FINDINGS OF THE PRESENT WORK

• Harpin$_{Pss}$-mediated yeast cell death was characterized:
  • Conditional expression of harpin$_{Pss}$ caused YCD
  • Harpins have no extracellular effect on $S. cerevisiae$ Y187 cells
  • Harpin$_{Pss}$-mediated YCD was independent of the stage of cell cycle
  • Chromosomal condensation and nuclear fragmentation does not seem to occur in harpin$_{Pss}$-induced YCD
  • Possible loss of membrane integrity in harpin$_{Pss}$-induced YCD was observed
  • ‘Petite’ mutants being insensitive to harpin$_{Pss}$-induced YCD indicate the possible role of mitochondria in harpin$_{Pss}$-induced YCD
  • No evidence for the leakage of Cyt C in harpin$_{Pss}$-induced YCD
  • The observed cell death in $S. cerevisiae$ Y187 was observed in Sey6211 and BJ2168 and the remaining three strains DY150, W303 and BY4741 were insensitive to harpin$_{Pss}$-induced YCD

• Structure-function relationship
  • Full-length $hrpZ_{psp}$ and $hrpZ_{Pst}$ caused YCD similar to $hrpZ_{Pss}$
  • Deletion mutation revealed that harpin$_{Pss}$ is a unique protein that retains the biological activity even in the 13 a.a. peptide (towards the C-terminal end), and any portion of the $hrpZ$ cause YCD
  • Sequence analysis revealed that harpin shares no homology with the known proteins whose structure was elucidated
  • The predicted secondary structure of harpin is helical in nature
  • Bio-informatic study revealed harpin$_{Pss}$ to have features of Type III chaperones
  • Harpin$_{Pss}$ crystallization was successful.