Chapter 3a

Involvement of Vitamins as downy mildew disease resistance activator in pearl millet to Sclerospora graminicola

3a.1. Introduction

Plant possesses a wide range of defenses that can be actively expressed in response to pathogen attack, insect herbivore and also to abiotic stresses. Of the many natural defense mechanisms, plants have evolved to survive in nature, only a few can trigger by biotic and abiotic agents without harmful effects (Ahn et al., 2005; Block et al., 2005). The timing of these defense responses is critical and there can be a difference between being able to cope or succumb to the challenge of a pathogen. During induction of systemic resistance (ISR), plant defenses are preconditioned by prior infection or treatment, which results in resistance against subsequent challenge by a pathogen or parasite. Plant immunization is a natural, safe, effective, eco-friendly and durable method to manage plant disease (Van Wees et al., 1997; Von Rad et al., 2005). It finds its niche in sustainable agriculture as one of the variety of practices that promote plant health and reduce the diseases of plant, thereby reducing the use of pesticides. Since induced resistance offers a significant potential for control of plant diseases, it has been extended to control disease resistance and was demonstrated in a number of plant pathosystems by using various abiotic and biotic inducers (Andreau et al., 2006).

The compounds or agents that activate the induction of resistance are referred to as inducers or elicitors, which can be biotic or abiotic in nature. Though several chemical compounds were identified as effective resistance inducers only a few like acibenzolar-S-methyl ester (ASM), probenazole (Yoshioka et al., 2001), benzathiadiazole (BTH; Oostendorp et al., 2001), actigard and messenger (Obradovic et al., 2005) were commercialized. Chemical plant defense activators have several advantages over disease control methods that depend on traditional fungicides or herbicides and breeding for disease resistance. Although many abiotic and biotic inducers have been tried and tested to identify a suitable one, there is still a gap in our knowledge in knowing the efficacy of several inducers. Among the various vitamins, only a few vitamins have been used as plant defense activators, therefore there is a need to study other vitamins as disease resistance activators effect on pearl millet and their ability in inducing resistance against downy mildew.
In recent years, the importance of vitamins as disease control agents and nutrients has been emphasized (Beyer et al., 2002; Pavet et al., 2005). Vitamins like menadione sodium bisulphite (Borges et al., 2003a), roseoflavin and riboflavin (Aver’yanov et al., 2000; Dong and Beer 2000) have been demonstrated to act as inducers of plant disease resistance against an array of plant pathogens. Menadione sodium bisulphite has been reported to induce resistance against panama disease in banana (Borges-Perez and Fernandez-Falcon, 1996). Menadione sodium bisulphite treatment has also induced resistance in B. napus to infection by L. maculans, causing phoma canker (Borges et al., 2003b). Vitamin B₁ induces disease resistance and functions as an activator of disease resistance in rice, A. thaliana and some vegetable crops to fungal, bacterial and viral infection, and also thiamine treatment has induced the transient expression of PR genes in rice and A. thaliana through the salicylic acid and related signalling pathway (Ahn et al., 2005). However, there are no reports for the vital role of these vitamins in inducing disease resistance against oomycete pathogens.

Hence, in the present study we have taken up to test ability of the major vitamins, vitamin K analogue MSB (Vit K₃), and the vitamin B analogues such as folic acid (Vit B₂), riboflavin (Vit B₂), niacin (Vit B₃), thiamine (Vit B₁), D-biotin (H) and pyridoxine (Vit B₆) were tested for their ability to induce downy mildew disease resistance and growth promotion in pearl millet.
3a.2. Materials and Methods

3a.2.1. Host

Seeds of pearl millet cultivar 7042S (susceptible to downy mildew disease) and IP18192 (resistant) were obtained from International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patencheru, India, under material transfer agreement and were used throughout the study.

3a.2.2. Pathogen and Inoculum Preparation

The downy mildew pathogen, *Sclerospora graminicola* was isolated from its susceptible host and maintained under greenhouse conditions at 22±2°C with 80 % relative humidity. Infected pearl millet leaves were collected in the evening and washed with water to remove the remnants of the earlier sporulation, and then leaf surfaces were blot-dried. The leaves were placed in petridishes lined by moist blotters and incubated in humidity chambers overnight. The sporangia were harvested next morning into sterile distilled water for the release of zoospores. The concentration of the zoospores in the suspension was adjusted to $4 \times 10^4$ zoospores/ml with sterile distilled water using haemocytometer as detailed by Singh *et al.* (1997).

3a.2.3. Vitamins Used in the Experiment

Vitamin K analogue menadione sodium bisulphite was obtained from Sigma Aldrich Chemical Co., (St. Louis, MO, USA), biotin procured from BDH Chemicals Ltd (Poole, England), folic acid and riboflavin, niacin, thiamine and pyridoxine were obtained from Hi media (Mumbai, India).

3a.2.4. Effect of Vitamins on Asexual Spores of *Sclerospora graminicola* to Study the Fungal Toxicity

To study the effect of vitamins on sporulation of *S. graminicola*, downy mildew infected leaves from the susceptible cultivar were collected, the existing sporangia were washed, surface wetness was removed and $1 \ cm^3$ leaf area was smeared with different dilutions of selected vitamin solutions at concentration of 10, 15, 20 and 25 mM for 30 min. Sterile distilled water treatment to infected leaves
served as control. Leaves were incubated in moist chambers for 12 h and observed for sporulation, sporangia from each treatment were harvested in one ml sterile distilled water and spore load was assessed using a haemocytometer.

3a.2.5. Inhibition of Sclerospora graminicola Zoospore Release and Motility

Sporangia suspension of S. graminicola prepared as per the procedures of Safeeulla (1976) and adjusted to 1.5x10⁴ sporangia ml⁻¹ using a haemocytometer and it was treated with different dilutions of selected vitamin solutions at concentrations of 10, 15, 20 and 25 mM for 15 min under dark conditions. Observations were made for zoospore release by counting the empty and intact sporangia. Zoospore suspension of S. graminicola was treated with test vitamins for 15 min and zoospore motility was observed and calculated based on number of zoospores showing motility in each vitamin treatments and rated as 100 % (+++), ≥ 50 % (++), ≥ 25 % (+) and immotile (-) based on proportion of spore showing sign of motility. A minimum of five microscopic fields was observed in three independent experiments for each treatment.

3a.2.6. Effect of Vitamin Treatment to Pearl Millet Seeds on Germination and Seedling Vigor under Laboratory Conditions

Pearl millet seeds of cv. 7042S were surface sterilized with 0.02% sodium hypochloride and two grams of seeds were soaked in 20 ml of the selected vitamin solutions at concentrations of 10, 15, 20 and 25 mM. Seeds were soaked in vitamin solutions for 6 h at 25±2°C with constant shaking and seed treatment with distilled water served as control. After soak treatment, the seeds were blotted dry and subjected to a germination test by paper towel method (ISTA, 2003). Seed quality evaluation parameters like percentage germination, root-length and shoot-length were recorded and vigor index (VI) was calculated for the seven-day-old seedlings by using the method of Abdul Baki and Anderson (1973). The experiment was carried out with four replicates of 100 seeds each.

\[ \text{VI} = \text{Percent germination} \times [\text{Mean root length} + \text{Mean shoot length}] \]
3a.2.7. Effect of Vitamins on Pearl Millet Plants against Downy Mildew Disease under Greenhouse Conditions

Pearl millet seeds were treated with different vitamins at above said concentrations for 6 h and were sown separately in clay pots containing 1:1:1 soil, sand and FYM (Fenu Yard Manure). Apron 35SD (a metalaxyl based seed-formulation) was also used as seed treatment at 6 g kg\(^{-1}\) to compare the vitamin seed treatment disease control efficiency, as it is the best available control measure for pearl millet downy mildew disease. Two-day-old-seedlings were inoculated to the whorl region of the coleoptile with a zoospore suspension of *S. graminicola* at a concentration of 4x10\(^4\) zoospores ml\(^{-1}\) by following the procedure of Singh and Gopinath (1985). These pots were arranged in a randomized complete block design and maintained under greenhouse conditions at 25±2ºC, 95 % relative humidity. The plants were rated for disease when they showed any one of the typical downy mildew symptoms such as sporulation on the abaxial leaf surface, chlorosis and stunted growth. The downy mildew disease incidence was recorded at 30 days after the emergence of seedlings. The experiment was carried out in four replicates of 100 seedlings. The scoring was done based on number of infected plants in total number of plants and expressed in percentage using the formula,

$$\text{Disease incidence (\%)} = \frac{\text{Total number of infected plants} \times 100}{\text{Total number of plants}}$$

The scoring system consisted of four level scale described as 0-5 % = highly resistant, 5.1-10 % = resistant, 10.1-25 % = susceptible and 25.1-100 % = highly susceptible (Sudhisha *et al.*, 2008). Percent protection offered was calculated using the formula,

$$\text{Percent protection} = \frac{\% \text{ downy mildew (DM) in untreated plants} - \% \text{ DM in vitamin-treated plants}}{\% \text{ downy mildew in untreated plants}} \times 100$$
3a.2.8. Optimization of Time Required for Vitamins to Induce Downy Mildew Disease Resistance

The nature of protection offered by vitamins was studied by maintaining spatio-temporal separation of the inducer treatments and the pathogen inoculation. Seed treatment with vitamins followed by raising the seedlings were the same as described earlier (3a.2.7). Spatial separation of 1, 2, 3, 4, 5, and 6 days was maintained between seedling emergence and pathogen inoculation i.e., the first set of vitamin-treated seedlings were inoculated at the coleoptile stage on day one followed by inoculation on the second, third, fourth, fifth and sixth day on separate set of seedlings and maintained under greenhouse conditions. The observation for disease was done at every 15 days time intervals and final data was recorded and tabulated at number of diseased plants was recorded at 30 day after emergence of the seedlings. The experiments were carried out in four replicates of 100 seedlings each and repeated twice.

3a.2.9. Durability of Disease Resistance

To determine the duration of protection conferred by vitamins, plants raised from vitamin-treated seeds, were challenge-inoculated twice with the downy mildew pathogen to confirm the systemicity and durability of induced resistance. The first inoculation was performed on two-day-old seedlings and the second to the whorls of nodal tillers (25-27 day-old-plants) and the third inoculation was performed to the inflorescence primordia (36-38 day-old-plants) at the boot leaf stage of the plants raised from vitamin treated seeds. Incidence of the disease and protection was recorded and calculated at 60 days after seed sowing.

3a.2.10. Effect of Vitamins on Pearl Millet against Downy Mildew Disease under Field Conditions

The vitamin treatments, which gave protection against downy mildew disease under greenhouse conditions, were further evaluated under field conditions by seed treatment, foliar spray and seed treatment followed by foliar spray. Trials were conducted at the downy mildew sick plot, which has been naturally infested with oospores of S. graminicola for three decades and provides the primary and
secondary inoculum from infector rows. The plants were raised by following the normal agronomical practices and disease screening was done using the infector row system (Williams et al., 1981). Disease incidence was recorded at 15 day-intervals and the data was computed finally at 60 days after sowing and plants were rated as diseased when they showed the typical downy mildew disease symptoms (Williams, 1984). Percent protection was calculated using the above mentioned formula.

3a.2.11. Mode of Vitamin Treatment

Seed Treatment

Seed treatment with vitamins was carried out as described earlier in the same chapter in section 3a.2.4. The vitamins used were thiamine, MSB, riboflavin, biotin and niacin at best concentration as these treatments were found to be effective under greenhouse conditions. Seed treatment with metalaxyl Apron 35SD at 6 g kg⁻¹ was also carried to compare the efficacy of vitamin treatment under field conditions.

Foliar Spray

To test the efficacy of foliar sprays, seven-day-old seedlings raised from untreated seeds were sprayed with a 20 mM concentration of each selected vitamin treatment using a portable hand sprayer till run off.

Seed Treatment Followed by Foliar Spray

Vitamin treated seeds were sown in the field as described earlier and also the sown seedlings were sprayed with the different selected vitamin solutions at the seven-day-old stage of pearl millet seedlings.

3a.2.12. Effect on Vegetative Growth and Reproduction of Pearl Millet by Vitamins

To test the effective role of vitamins in promoting vegetative growth and reproductive parameters, susceptible pearl millet seeds were treated with the promising vitamins and sown in the field. The effects were measured by recording the plant height, length and girth of ear head at 60 days. 1000 seed weight and yield were determined at the time of harvest (Williams and Singh, 1981).
3a.2.13. Statistical Analysis

All replicates data from laboratory, greenhouse and field experiments were analyzed separately for each experiment and subjected to arcsine transformation and analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by P values ($P \leq 0.05$). Treatment means were separated by Tukey’s Honestly Significant Differences (HSD) test.
3a.3. Results

3a.3.1. Effect of Vitamins on Asexual Spores of Sclerospora graminicola to Study the Fungal Toxicity

Vitamins evaluated in this study as inducers did not show any fungal toxic effect on \textit{S. graminicola} at any of the tested vitamin concentrations. There was no inhibition of the asexual sporulation of \textit{S. graminicola}, since the leaf bits treated with the test inducers showed profuse sporulation where as in the leaf bits treated with metalaxyl there was complete inhibition was observed. Similarly, all the tested vitamins treatments did not have any effect on the release of zoospore from sporangia at all the concentration tested. Complete inhibition of zoospore release was recorded in metalaxyl treatment (positive control). In control set there was no inhibition of release of zoospores (Figure 3a.1 and Table 3a.1). Hence the different vitamin concentrations used, did not show any toxic effect on sporulation and as well as release of zoospores of \textit{S. graminicola}.

3a.3.1a. Vitamin Treatments and Seed Germination and Seedling Vigor

Vitamin seed soak treatments for 6 h at 20 mM concentration significantly enhanced the seed germination and seedling vigor of pearl millet (Table 3a.1) hence the 20 mM concentration was selected for further studies. Maximum germination of 93\% and seedling vigor of 1318 was observed in thiamine treatment followed by MSB soak treatment which resulted in 93\% germination and seedling vigor of 1312. Niacin, pyridoxine, biotin and folic acid were also found to enhance both germination and vigor albeit to a lesser extent than thiamine and MSB. However, treatments of thiamine, MSB, niacin, riboflavin and biotin were found to be more effective in enhancing seed germination than any of the vitamins used. The untreated seeds had 88\% germination with 990-seedling vigor, lower than vitamin treated seeds (Table 3a.1). There is no statistical difference ($P<0.05$) between the thiamine, MSB treated susceptible pearl millet seedlings when compared to the apron treatment which resulted in 93\% germination with 1470 seedling vigor.
Table 3a.1: Influence of Seed Treatment with Vitamins on Seed Germination, Vigor Index in Pearl millet and Anti-mildew activity on *Sclerospora graminicola*.

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Vitamin treatments</th>
<th>Concentration (mM)</th>
<th>Pearl millet seed germination (%)</th>
<th>Pearl millet seedling Vigor Index</th>
<th>Sporulation of asexual spores of <em>S. graminicola</em></th>
<th>Zoospores release from sporangia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MSB</td>
<td>10 mM</td>
<td>90 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1290 ± 5.196&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td></td>
<td></td>
<td>15 mM</td>
<td>91 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1198 ± 1.15&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td></td>
<td></td>
<td>20 mM</td>
<td>93 ± 0.577&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1312 ± 21.36&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>+++</td>
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<tr>
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<td></td>
<td>25 mM</td>
<td>91 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1290 ± 51.96&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>Riboflavin</td>
<td>10 mM</td>
<td>90 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1190 ± 23.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td></td>
<td></td>
<td>15 mM</td>
<td>91 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1154 ± 16.74&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td></td>
<td></td>
<td>20 mM</td>
<td>92 ± 1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1223 ± 13.27&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<td></td>
<td></td>
<td>25 mM</td>
<td>91 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1185 ± 23.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>Folic acid</td>
<td>10 mM</td>
<td>89 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1055 ± 31.75&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td></td>
<td></td>
<td>15 mM</td>
<td>90 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1060 ± 46.39&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>+++</td>
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<td></td>
<td></td>
<td>20 mM</td>
<td>90 ± 1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1070 ± 11.54&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>+++</td>
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<td></td>
<td></td>
<td>25 mM</td>
<td>90 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1020 ± 5.77&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>+++</td>
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<tr>
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<td>Niacin</td>
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<td>91 ± 1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1185 ± 34.64&lt;sup&gt;b&lt;/sup&gt;def&lt;sup&gt;ef&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>15 mM</td>
<td>91 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1160 ± 31.75&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<td>25 mM</td>
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<td>1209 ± 5.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
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<td>Pyridoxine</td>
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<td>89 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1176 ± 15.01&lt;sup&gt;cdef&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<td></td>
<td></td>
<td>15 mM</td>
<td>90 ± 0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1190 ± 8.66&lt;sup&gt;b&lt;/sup&gt;def&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<td></td>
<td></td>
<td>20 mM</td>
<td>91 ± 1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1210 ± 11.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++</td>
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<tr>
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<td>25 mM</td>
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<td>1175 ± 14.43&lt;sup&gt;cdef&lt;/sup&gt;</td>
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<td>D-biotin</td>
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<td>1095 ± 82.52&lt;sup&gt;efgh&lt;/sup&gt;</td>
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<td></td>
<td>25 mM</td>
<td>91 ± 1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1121 ± 12.12&lt;sup&gt;efgh&lt;/sup&gt;</td>
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<tr>
<td>7</td>
<td>Thiamine</td>
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<td>92 ± 1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1279 ± 16.74&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>15 mM</td>
<td>92 ± 1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1290 ± 8.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
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<td></td>
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<td>20 mM</td>
<td>93 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1318 ± 10.39&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>25 mM</td>
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<tr>
<td>8</td>
<td>Control</td>
<td></td>
<td>88 ± 1.150&lt;sup&gt;b&lt;/sup&gt;</td>
<td>990 ± 5.77&lt;sup&gt;i&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>9</td>
<td>Apron</td>
<td></td>
<td>93 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1470 ±17.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No sporulation</td>
<td>No sporulation</td>
</tr>
</tbody>
</table>

Note: +=25%, ++=50%, +++=100% sporulation indicates the sporulation and zoospore release from the sporangia. Means with different superscripts are significantly different from each other as indicated by Tukey’s HSD (P< 0.05).
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3a.3.2. Seed Treatment with Vitamins and Effect on Pearl millet Downy Mildew Disease under Greenhouse Conditions

Seed treatment with vitamins protected pearl millet seedlings against downy mildew disease when compared to the untreated control. However, Apron seed treatment was found to be the most effective as it afforded 92% downy mildew disease protection. Seed treatment with MSB resulted in 71% protection followed by niacin and riboflavin, which offered 63 and 62% protection respectively. Biotin, pyridoxine and folic acid treatment gave 51, 44 and 42% protection respectively (Figure 3a.2 & 3a.3). However, the highest degree of disease protection was observed in seeds treated with thiamine (73%). Although the treatment of MSB and thiamine resulted difference in the degree of protection, there was no statistical difference ($P\leq0.05$) between the protection offered by treatments of MSB and thiamine.

Due to the low efficacy of folic acid and pyridoxine in protecting pearl millet seedlings against downy mildew disease, these treatments were not considered for the subsequent experiments including the field trials.
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Figure 3a.2: Greenhouse Experiment Showing Downy Mildew Disease Protection after Inoculation with *Sclerospora Graminicola* following Seed Treatment of Pearl Millet with Vitamins. The bars indicate standard error. Means with different letters are significantly different from each other as indicated by Tukey’s HSD ($P \leq 0.05$)

Figure 3a.3: Effect of Seed Treatment with Different Concentrations of Thiamine following Artificial Inoculation on Downy Mildew Pathogen under Greenhouse Conditions.

**Control**- Pearl millet crop resulted from untreated seeds and showing heavy incidence of downy mildew disease.

**Thiamine (5 mM)** - Pearl millet crop resulting from the Thiamine treated seeds at 5 mM

**Thiamine (10 mM)** - Pearl millet crop resulting from the Thiamine treated seeds at 10 mM

**Thiamine (15 mM)** - Pearl millet crop resulting from the Thiamine treated seeds at 15 mM

**Thiamine (20 mM)** - Pearl millet crop resulting from the Thiamine treated seeds at 20 mM

**Thiamine (25 mM)** - Pearl millet crop resulting from the Thiamine treated seeds at 25 mM

**Resistant**- Pearl millet crop resulting from the Apron 35 SD treated seeds and the protection against downy mildew disease.
3a.3.3. Vitamin Treatments to Seeds and Seedling Inoculation with the Downy Mildew Pathogen at Different Time Intervals for Spatial and Temporal Effects in Inducing Disease Resistance

Two-day-old emerging seedlings were inoculated in the whorl region of the coleoptiles with a zoospore suspension of \textit{S. graminicola} with time gaps of 1, 2, 3, 4, 5 and 6 days. Seedlings inoculated on the fourth day of the pathogen followed by challenge inoculation demonstrated maximum protection against downy mildew disease. The protection was found to be stable thereafter as at the fifth and sixth day, the resistance induced was maintained at the same levels (Figure 3a.4).

Figure 3a.4: Vitamin Seed Treatments to Pearl Millet and the Spatio-Temporal Effect in Induction of Resistance. The experiment in this study was carried out in four replicates of 100 seedlings each and the bars indicate standard error. Means with different letters are significantly different from each other as indicated by Tukey’s HSD ($P \leq 0.05$).
3a.3.4. Durability of Downy Mildew Resistance Induced by Vitamins during the Crop Growth Period

Disease protection to nodal tillers including the main shoot tip and inflorescence primordial of vitamin-treated plants inoculated with the downy mildew pathogen remained at the same level as at the seedling stage. Plants raised from vitamin seed treatment with a thiamine had a maximum 80 % protection, while MSB offered 77 %. The other vitamins like biotin, niacin and riboflavin offered 73, 69 and 64 % disease protection respectively at nodal tillers period. At the inflorescence primordial stage, the inoculation with the pathogen of plants raised from seed treated with thiamine offered 81 % disease protection followed by MSB (79 %). Plants raised from seed treated with the biotin treatment showed 75 % but niacin and riboflavin exhibited 71 and 69 % disease protection respectively (Table 3a.2).

Table 3a.2: Downy Mildew Disease Incidence (DMDI) in Nodal Tillers and Inflorescence under Greenhouse Conditions. Pearl Millet Plants raised from Seeds Treated with Vitamins and Challenge Inoculated with Sclerospora Graminicola.

<table>
<thead>
<tr>
<th>Vitamin seed treatments</th>
<th>Inoculation with <em>Sclerospora graminicola</em> to the pearl millet nodal tillers</th>
<th>Inoculation with <em>Sclerospora graminicola</em> into the Inflorescence primordial at boot leaf stage of pearl millet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent Protection</td>
<td>Per cent Protection</td>
</tr>
<tr>
<td>1. MSB</td>
<td>77 ± 1.38</td>
<td>79 ± 3.16</td>
</tr>
<tr>
<td>2. Niacin</td>
<td>69 ± 1.38</td>
<td>71 ± 2.10</td>
</tr>
<tr>
<td>3. Riboflavin</td>
<td>64 ± 2.75</td>
<td>69 ± 0.57</td>
</tr>
<tr>
<td>4. Thiamine</td>
<td>80 ± 0.57</td>
<td>81 ± 1.38</td>
</tr>
<tr>
<td>5. Biotin</td>
<td>73 ± 2.75</td>
<td>75 ± 2.75</td>
</tr>
<tr>
<td>6. Un-treated Control (Challenged)</td>
<td>7± 2.75</td>
<td>8± 2.75</td>
</tr>
</tbody>
</table>

Note: Means with different superscripts are significantly different from each other as indicated by Tukey’s HSD (P ≤ 0.05).
3a.3.5. Vitamin Treatments and Downy Mildew Disease Reaction under Field Conditions

3a.3.5a. Seed Treatment

Seed treatment with thiamine and MSB offered 71 and 70 % downy mildew disease protection respectively (Figure 3a.5 and Figure 3a.6). Seed treatment with biotin offered 68 % protection against downy mildew disease followed by niacin and riboflavin, which offered 64 and 61 % protection respectively. However, Apron treatment (positive control) showed the best downy mildew disease protection with 91 % protection and no disease protection was observed in untreated seeds (control).
3a.3.5b. Foliar Spray

The foliar spray to seven-day-old seedlings from seeds treated with thiamine recorded 68 % protection. In seed-soak treatments, the protection offered by MSB was 70 % whereas the same vitamin showed lesser protection of 66 % by foliar spray. Whereas biotin offered 63 % protection and treatment with riboflavin and niacin offered 59 and 61 % respectively (Figure 3a.5). However, foliar spray was found to be less effective than seed treatment with vitamins in protecting pearl millet against downy mildew disease.

Figure 3a.5: Field Demonstration of Effect of Thiamine Seed Treatments in Pearl Millet Protecting against Downy Mildew Disease.

Control- Pearl millet crop resulted from untreated seeds and showing heavy incidence of downy mildew disease.

Thiamine (5 mM) – Pearl millet crop resulting from the Thiamine treated seeds at 5 mM
Thiamine (10 mM) – Pearl millet crop resulting from the Thiamine treated seeds at 10 mM
Thiamine (15 mM) – Pearl millet crop resulting from the Thiamine treated seeds at 15 mM
Thiamine (20 mM) – Pearl millet crop resulting from the Thiamine treated seeds at 20 mM
Thiamine (25 mM) – Pearl millet crop resulting from the Thiamine treated seeds at 25 mM
Resistant- Pearl millet crop resulting from the Apron 35 SD treated seeds and the protection against downy mildew disease.
3a.3.5c. Seed Treatment followed by Foliar Spray

Seed treatment followed by a foliar spray with vitamins was found to be the most effective in inducing downy mildew disease protection as it offered higher levels of protection than seed treatment or foliar spray alone. Vitamin seed treatments followed by a foliar spray to seven-day-old seedlings with thiamine offered 74% protection compared to MSB which showed 72% protection followed by a biotin with 71%, riboflavin and niacin offered 67 and 65% respectively (Figure 3a.6). The seed treatment and foliar spray applications showed similar downy mildew protection in pearl millet, but seed treatment followed by foliar application was better ($P \leq 0.05$) when compared to seed treatment or foliar spray alone.

![Bar graph showing vitamin treatments and their percent protection against downy mildew disease.](image)

**Figure 3a.6:** Evaluation of Vitamins to Induce Resistance in Pearl Millet against Downy Mildew Disease under Field Conditions using Different Modes of Vitamin Application. The experiment in this study was carried out in four replicates of 100 seedlings each and the bars indicate standard error. Means with different letters are significantly different from each other as indicated by Tukey’s HSD ($P \leq 0.05$).
3a.4.6. Effect of Seed Treatment with Vitamins on Growth Parameters of Pearl Millet

Seed treatment with vitamins not only resulted in increased disease resistance against *S. graminicola*, but also significantly enhanced the growth parameters of the pearl millet plants. Plant height was increased by up to 13.5 cm and flowering was 1-4 day earlier than the untreated plants depending on the treatment. The number of productive tillers in untreated plants was two whereas thiamine treatment increased the mean to 3.5. It also enhanced the mean of the length of the ear head from 9.4 cm in control plants to 12.4 cm in thiamine-treated plants. Mean of the girth of ears increased from 3.6 cm in control plants to 4.3 cm in plants treated with thiamine.

In addition, 1000 seed weight and yield increased in all the vitamin-treated plants compared to the untreated plants. The mean value of 1000 seed weight was 7.4 g in untreated plants whereas in thiamine-treated plants it was 11.92 g. Similarly, treatment with thiamine also enhanced the yield from 1209 kg ha⁻¹ in control plants to 1368 kg ha⁻¹ which showed the highest yield (Table 3a.3).
Table 3a.3: Effect of Seed treatment with Vitamins on Growth Parameters of Pearl Millet Plants

<table>
<thead>
<tr>
<th>Vitamin seed treatments</th>
<th>Growth Parameters</th>
<th>Plant height (cm) a</th>
<th>Days required for 50% flowering b</th>
<th>Total number of productive tillers per plant c</th>
<th>Length of ears (cm) d</th>
<th>Girth of ears (cm) e</th>
<th>1000 seed weight (g) f</th>
<th>Yield (kg per ha) g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MSB</td>
<td></td>
<td>68±1.73ab</td>
<td>40±0.57a</td>
<td>3.4±0.05ac</td>
<td>12±0.57a</td>
<td>4.1±0.5ab</td>
<td>11.7±0.08a</td>
<td>1328±15.5a</td>
</tr>
<tr>
<td>2. Niacin</td>
<td></td>
<td>66±1.15ab</td>
<td>42±1.15a</td>
<td>3.2±0.11a</td>
<td>11.8±0.11a</td>
<td>4±0.11ab</td>
<td>10.97±0.5a</td>
<td>1306±3.4ab</td>
</tr>
<tr>
<td>3. Riboflavin</td>
<td></td>
<td>62±0.57bc</td>
<td>43±0.6a</td>
<td>3.0±0.05a</td>
<td>11.4±0.23a</td>
<td>3.9±0.2ab</td>
<td>9.5±0.28ab</td>
<td>1281±17.3ab</td>
</tr>
<tr>
<td>4. Thiamine</td>
<td></td>
<td>72±1.17a</td>
<td>40±1.15a</td>
<td>3.5±0.23a</td>
<td>12.4±0.23a</td>
<td>4.3±0.1ab</td>
<td>11.92±1.1a</td>
<td>1368±21.93a</td>
</tr>
<tr>
<td>5. Biotin</td>
<td></td>
<td>64±2.3bc</td>
<td>42±1.73a</td>
<td>3.0±0.11a</td>
<td>11.6±0.34a</td>
<td>4.0±0.057ab</td>
<td>9.7±0.11ab</td>
<td>1231±17.8b</td>
</tr>
<tr>
<td>6. Control</td>
<td></td>
<td>58.5±0.2c</td>
<td>44±0.6a</td>
<td>2.1±0.05b</td>
<td>9.4±0.23b</td>
<td>3.6±0.05b</td>
<td>7.4±0.23b</td>
<td>1209±33.7c</td>
</tr>
</tbody>
</table>

Note: Means with different superscripts are significantly different from each other as indicated by Tukey’s HSD (P < 0.05).

a Mean of two-repeated experiment with 50 plants with four replications in each treatment.
b Number of days taken by 50% of the total number of plants in each replicate to flower.
c Number of basal tillers produced by each plant.
d Measured from the base to the tip of the ear head using measuring tape.
e Measured as the circumference of the ear head at the centre using measuring tape.
f Calculated by weighing 1000 seeds in eight replicates (ISTA, 2003).
g Based on the weight of the seeds collected from the central two replicates and converting it into one hectare (Williams and Singh, 1981).
MSB-Menadione sodium bisulphite.
3a.4. Discussion

Vitamins are produced by animals, plants and micro-organisms and act as coenzymes in many physiological processes (Gregory, 1998; Fischer and Bacher, 2006). Vitamin-based commercial formulations such as Binary CQ, Nucol, Organic Gardening and Remit are designed to enhance foliar nutrients, which help in improving the crop quality and productivity by increasing the size and quality of the plant yield and also by offering protection against diseases. Foliar application of riboflavin effectively controlled several diseases of *N. tabaccum* (Dong *et al*., 1995) and it reduced the powdery mildew disease of *Fragaria virginiana* in combination with methionine, metal ions and a surfactant (Wang and Tzeng, 1998). Riboflavin at 1 mM treatment caused induction of systemic resistance in chick pea against fusarial wilt and charcoal rot disease. At this concentration, riboflavin neither caused cell death of the host plant directly affected the pathogen growth (Saikia *et al*., 2006).

Vitamin K₃ has recently been shown to induce resistance against panama disease in banana (Borges-Perez and Fernandez-Falcon, 1996; Borges *et al*., 2004) and in *B. napus* to infection by *L. maculans* (Borges *et al*., 2003b). In another research, Emmanouil and Wood (1981) observed that treating the leaves of pepper, tomato or egg plant with riboflavin prior to inoculation of roots with *Verticillium dahliae* significantly reduced the fungal load and overall disease symptoms of these plants. Exogenous application of riboflavin and roseoflavin were also shown to protect susceptible rice from blast disease and to induce fungal toxicity mediated by active oxygen (Aver’yanov *et al*., 2000).

Apron 35SD, a metalaxyl seed treatment formulation is currently recommended for downy mildew disease control in pearl millet at 6 g kg⁻¹. However, metalaxyl is expensive and not yet made available to poor farmers in India. It has already been reported that oomycete pathogen are showing metalaxyl resistance and hence the search for cheaper commercial formulations of effective compounds are required (Thakur and Mathur, 2002). But the disease protection provided by the fungicides is limited to approximately 35 days of crop growth, but secondary and tertiary tillers of pearl millet are produced for several weeks and
remain susceptible up to 65 to 70 days after sowing. Therefore, repeated curative control measures with foliar sprays are essential for ensuring long lasting control of downy mildew disease. However, multiple or even single use of metalaxyl is not feasible for a low value high volume crop and farmers cannot afford such measures for disease control (Jeger et al., 1998). In addition, reduced sensitivity to metalaxyl has seriously set back chemical control of downy mildew (Cooke and Less, 2004). In such a scenario, alternative downy mildew control measures like induction of resistance with vitamins offers durable, and an attractive proposal for poor farmers.

Under greenhouse conditions, seed treatment with different vitamins reduced downy mildew disease and offered various levels of protection. The vitamin treatment offered induced resistance to pearl millet downy mildew pathogen even after production of nodal tillers and inflorescence. Thiamine treatment at 20 mM is sufficient to protect the crop against downy mildew disease. Treatment with MSB and niacin were showed similar downy mildew disease protection. However, the induction of resistance by thiamine application has been reported by various other workers. The disease progress inhibiting activities of thiamine against \textit{Xanthomonas oryzae} in rice, pepper mild mottle virus infection in tobacco plants, \textit{Colletotrichum lagenarium} in cucumber and virulent \textit{P. syringe} in \textit{Arabidopsis} plants has been proved. These results imply that thiamine induces resistance in plants to infection by various bacterial, viral and fungal pathogens (Ahn et al., 2005).

Induced systemic resistance is expressed as spatial and temporal separation between inducer treatment and challenge inoculation for activation of defense responses (Ryals et al., 1996). In our studies, a one-day interval between vitamin treatment and challenge inoculation did not give significant protection, but a two-day interval gave significant protection for most vitamins. However, a four-day interval between inducer treatment and challenge inoculation was optimal for resistance buildup, indicating that this is the time necessary for activation of a level of defense, sufficient to inhibit the pathogen growth and suppress the disease.
Protection offered by the 5 and 6 days interval treatment was statistically similar to that of the day four interval.

Advancing from greenhouse trials to field trials is an important step towards the goal of practical applications of induced resistance elicited by any biotic or abiotic agents. The vitamins showing good performances under greenhouse conditions were further evaluated under epiphytotic conditions in the field. Borges et al. (2004) have reported that panama wilt of banana was effectively controlled by foliar application of MSB. The difference between the effectiveness in protecting plants by foliar sprays and seed treatment may be due to the leaf lamina of *A. thaliana* a dicotyledonous, and banana, although a monocotyledon plant with broad leaf. Pearl millet is a monocotyledon with a lanceolate leaf and these plants also differ in their ontogeny, morphology and physiological status, which might make a difference to vitamin absorbance. Since both seed treatment and foliar spray effectively protect pearl millet, seed treatment alone can be adopted as feasible technique for poor marginal farmers. To make foliar spray application effective, it requires proper equipment, spray volume, plant coverage, timing and manpower which again increase the expense to farmers.

Vitamins act as cofactors of enzyme flavoproteins, some of which catalyze lipid peroxidation, a main process in producing reactive oxygen intermediates (ROIs) that serve as a signaling network in plant immune responses (Alvarez et al., 1998; Fischer and Bacher, 2006). Riboflavin is an activator of a novel-signaling pathway leading to systemic resistance. When, applied as foliar spray to *A. thaliana* against *P. parasitica* it increased peroxidation and antioxidative defense enzymes, resulting in activation of PR genes, suggesting that riboflavin initiated the resistance signal transduction (Dong and Beer, 2000). It would be interesting to study the mechanism of induced resistance offered by thiamine in order to identify new metabolic targets for intervention and expression for achieving higher downy mildew disease suppression ability.

Vitamin-treatments significantly enhanced seed germination and seedling vigor of pearl millet. Pearl millet plants showed increased height, number of tillers,
productive ears, and length of the ears, ear girth, 1000 seed weight and yield when compared with untreated control. The vitamins used in this study were not phytotoxic even at higher concentrations needed for induction of disease resistance. Vitamins are considered as bio-regulator compounds which in small concentrations exert a profound influence upon plant growth. Application of thiamine at 100 ppm and ascorbic acid 200 ppm enhanced the vegetative growth and flowering parameters in gladiolus plant (Nahed et al., 2009).

In another research, Karima et al., (2005) on sunflower plant found that application of ascorbic acid at 60 ppm led to significant increase in plant height, number of leaves, fresh and dry weight of leaves. Youssef and Talaat (2003) reported that pronounced increases in vegetative growth and chemical constituents of rosemary plants by foliar application of thiamine. Other investigators found similar results on the stimulatory effects of vitamin C on other plants such as on potato (El-Banna et al., 2006), pepper (Shehata et al., 2002) and pea plants (Helal et al., 2005). Also, Abdel-Halim (1995) found that the application of vitamin C on tomato plants caused significant increase on growth parameters (stem length, number of branches, leaves, flowers and fruit set and dry weight of shoots per plant) as well as total weight, number of fruits and total yield. Enhancing the efficacy of seed priming with vitamin-based formulations may help to increase induced systemic resistance in pearl millet, which in turn, would improve the plant growth and yield.

Thus, from the above investigation, among the vitamins tested, thiamine was found to be the best inducer to induce downy mildew disease to pearl millet at 20 mM concentration when compared to other vitamin treatments. Hence for further studies among vitamins, thiamine was selected at 20 mM concentration which was used in chapter 4 and chapter 5 experiments.