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Disease Incidence and Transmission of *Xanthomonas axonopodis* pv. *phaseoli* (smith) in Black Gram Grown in Western India

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Abstract

A total of 173 seed samples of black gram were collected from 21 districts in Rajasthan. The seeds of each sample were categorised into three categories, namely asymptomatic (28.25–93.25%), bold-discolored (02.00–52.75%) and shrivelled-discolored (02.25–36.50%) seeds. The pathogen was detected in 152 samples on PTS medium with an incidence range of 02-100% from 21 districts of Rajasthan. The bacterium was identified on the basis of various morphological, biochemical, and pathogenicity tests. Heavy infection of bacteria caused failure of seed germination and a heavy incidence of pathogens. The plants showed various disease symptoms of circular to irregular brown spots on the leaves. In severe cases, necrosis and blight of the apical parts of leaves were observed. Brown lesions and streaks were observed on the infected stem. At the time of fruiting, browning and rotting of pods were observed. Brown necrotic spots on cotyledonary leaves, hypocotyls, and seedling transition zones cause seedling mortality, which increases gradually over time.

Key words: Black gram seeds, *Xanthomonas axonopodis* pv. *phaseoli,* incidence, phytopathological effects, disease transmission.

1. INTRODUCTION

Black gram or urd [*Vigna mungo* (Linn.) Hepper], is the largest exported pulse crop attacked by various microbes that reduce the marketable price of the crop. The bacterium *Xantomonas axonopodis* pv. *phaseoli* is responsible for the fuscous blight disease in blackgram in Germany (Zochowski and Rudolph, 1991). This disease has also been reported from various parts of India, resulting in yield losses of up to 40% of black gram (Singh, Gurha and Ghosh, 1998, Singh, 2001, Mutlu et al. 2008). The disease is characterised by brown, dry raised spots on leaves, pods, and stems. When the disease is severe, the leaves become yellow and fall off prematurely. The stems and pods also get infected with disease severity (Singh *et al*. 1998).

In the present study, the incidence of the pathogen in seeds grown in Rajasthan state, India and the transmission of seed-borne inoculum from seed to plant was studied. Around 62.5 MT of the crop are exported around the world, which is 40% of the total amount of beans and pulses. It is grown on an area of about 3.1 million ha, producing 1.49 MT, accounting for 13% of the total pulse production of India. The crop is grown a lot in Rajasthan. It makes up 22% of all blackgram production in India (Anonymous, 2006, 2010).

2. MATERIAL AND METHODS

2.1 Identification and Incidence of the Pathogen

A total of 173 seed samples of black gram collected from 21 districts of Rajasthan were studied by dry seed examination, the standard blotter method (SBM) and the agar plate method (Anonymous, 1985) to determine the diseases associated with seeds and seedlings. A Pepton Tyrosine Starch (PTS) medium was used for the detection and incidence of *X. axonopodis* pv. *phaseoli* in seed samples. After incubation, typical bacterial colonies from seeds raised on nutrient agar were transferred to PTS agar medium to obtain a pure culture. The pure colonies of bacterium were subjected to various tests, namely Gram's staining, KOH solubility test, levan formation, oxidase tests (Kovac's, 1956, Hildbrand and Scroth, 1972), potato soft rot test, nitrate reductase test (Fahy and Persley, 1983), arginine dihydrolysis, gelatin hydrolysis test, hypersensitivity test in tobacco and pathogenicity tests (Lelliot and Stead, 1987) for the identification of the bacterial species.

2.2 Phytopathological effects and disease transmission

Phytopathological effects of the pathogen were studied by the Petri plate method, test tube seedling symptoms test, and pot experiment. The related data, such as seed germination, seedling symptoms, and mortality, were recorded timely. Two seed samples of black gram (ac. Nos. Vm-1104 and Vm-1119) carrying a 100% natural infection of *X. axonopodis* pv. *phaseoli* were selected. One hundred seeds per category per sample were sown on moist blotters (10 seeds/plate) and 1% water agar medium in test tubes (1 seed/test tube) and incubated at 25±2o C for 12–12 h, alternating cycles of light and darkness, up to 7 days and 14 days, respectively. In the pot experiment, 100 seeds per category per sample were sown in earthen or plastic pots (5 seeds/pot).

2.3 Patogenicity tests (host tests)

(a) Stab inoculation technique

Healthy seeds of blackgram were placed on moistened blotters in a Petri plate and incubated at 25±2°C for 5 days in darkness. The seedlings at staple stage were collected and inoculated by stabbing the cotyledons with a needle smeared with the test bacterial cells (10^8-10^9 cfu/ml at 600 nm). Observations were taken for symptoms produced on seedlings up to 7 days after inoculation.

(b) Seed smoothing

After rolling the seed in a 24 hour old test bacterial culture, healthy blackgram seeds (10 seeds/Petri plate) were placed on moistened blotters and incubated at 252°C in darkness. Observations were taken for symptoms on seeds and seedlings up to 7 days after the inoculation.

3. RESULTS AND DISCUSSION

3.1 Identification and incidence of the pathogen

All the 173 seed samples of black gram from 21 districts revealed asymptomatic (28.25–93.25%), bold-discolored (2.52–75%), and shrivelled-discolored (2.25–36.5%) seeds (Table 1, Fig. 1a–b). Seed lots infected with *X. axonopodis* pv. *phaseoli* prominently contained seeds that had brown discolorations, spots, and shrivelling as signs. Similar observations have been reported in infected seeds of *Phaseolus* sp. with wrinkled and discoloured hilum region due to *X. campestris* pv. *phaseoli* infection (Lelliot and Stead, 1987), in rape and mustard seeds with pink to brown water-soaked translucent areas on seed (Sharma, Agrawal and Singh, 1992), and in pigeon pea in which the discoloured seeds showed water-soaked translucent areas and pink discolorations due to *X. campestris* pv. *cajani* (Sharma et al., 2001).

In 165, 159, and 152 seed samples, the pathogen was found in untreated (3.34–90%) and pretreated (3.34–83.34%) on SBM and PTS agar medium (2-100%). Samples from Tonk (6-100%), Jaipur (20-98%), Bikaner (16-97%), Alwar (56-96%), Jodhpur (34-96%), Jhunjhunu (47-93%), Sriganganagar (42-93%), Chittorgarh (37-93%), Udaipur (21-92%), and Bundi (31-91%) showed relatively higher incidence of the pathogen (Table 1).

The bacterial colonies isolated from various seed samples produced yellow, mucoid colonies with a halo of hydrolysis around the colonies on PTS agar medium (Fig.1c-d) and were identified as *X. axonopodis pv. phaseoli.* The isolates were Gram's negative, KOH solubility test positive, levan negative, oxidase negative, arginin positive (Fig.1e), gelating hydrolyzing (Fig.1f), potato soft rot test positive, nitrate reductase test negative, and positive hypersensitivity reaction (HR) test caused by the pathogen on tobacco leaves after its infiltration, developing the brown necrotic symptoms on tobacco leaves (Fig.1g).

3.2 Phytopathological effects and disease transmission

Two seed samples of blackgram carrying 100% natural infection of the pathogen were selected for disease transmission studies.

3.2.1 Petri plate method

Radicle emergence started after 24 h of incubation and increased up to the 4th day. The maximum seed germination was on the 5th day of incubation, being 100% in both samples of asymptomatic; 91 and 94% in bold-discolored and 38 and 74% in shrivelled-discolored seeds in ac nos. Vm-1104 and Vm-1119, respectively. Ungerminated seeds showed rotting due to heavy growth of the bacterium on and around the seeds. Such ungerminated seeds were 0, 9 and 62% in sample Vm-1104 and 0, 6 and 25% in Vm-1119 in asymptomatic, bold-discolored, and shrivelled-discolored seed categories, respectively. Initially, the symptoms were reported as irregular brown spots and rotting of the radicle and plumule (Fig.1h). In three categories, the symptomatic seedlings were 31, 56.04, and 89.47% in ac no Vm-1104; and 28, 52.34, and 85.13% in ac no Vm-1119.The seedling mortality was 6, 9.89, and 44.74% in ac no Vm-1104 and 6, 6.38, and 12.16% in ac no Vm-1119 in the three categories, respectively (Fig. 2a).

3.2.2 Water agar test tube seedling symptoms test (TTSST)

On water agar, the seed germination was 100, 94, and 62% in ac no Vm-1104; and 100, 100, and 74% in ac no Vm-1119 in asymptomatic, bold-discolored, and shrivelled-discolored seed categories, respectively, on the 15th day of incubation. Initially, the symptoms appeared on the 3rd to 5th day of incubation. The seedlings exhibit bacterial oozing, small, brown, irregular water-soaked spots on their cotyledonary leaves. These heavily infected seedlings later showed rotting and the hypocotyls collapsed (Fig.1i). The symptomatic seedlings were 26, 89.19, and 100% in ac no Vm-1104; and 21, 89.18, and 92% in ac no Vm-1119 in the three categories of seeds, respectively, on the 15th day of incubation. Maximum mortality of seedlings on the 15th day was 87.09 and 52.70% in shrivelled-discoloured seeds in ac nos Vm-1104 and Vm-1119, respectively (Fig. 2b).

3.2.3 Pot experiment

The seed germination started during 5–10 days of sowing and was 100, 81, and 54% in ac no Vm-1104 and 100, 88, and 66% in ac no Vm-1119, respectively in all the three categories. The disease symptoms appeared as small, circular to irregular brown spots on the lower surface of the leaves within the 15-20th day of sowing (Fig.1j). The symptomatic seedlings were 14, 91.36 and 100% in ac no Vm-1104 and 14, 81.82 and 92.42% in ac no Vm-1119 in the three categories of both samples, respectively. The symptoms appeared on cotyledonary leaves, the hypocotyls collapsed, and the apical shoot showed rotting. Ultimately, such seedlings died. The mortality of seedlings due to heavy infection was 8, 77.78% and 83.95 in ac no Vm-1104 and 8, 38.64 and 69.70% in ac no Vm-1119, respectively in the three categories (Fig. 2c).

After 60 days of sowing in heavily symptomatic plants, the area between several large spots becomes dry and brown, or the edges of the leaf show twisting due to spots. The infected plants were weak and had comparatively smaller leaves showing brown spots, browning at the pedicle, poor development of

flowers and pods of small size. Infected plant parts incubated on agar media revealed the pathogen. The fruit pods have dark-colored, raised, cankerous growth on their surface that appears to be surrounded by water-soaked borders. In heavily symptomatic plants, the infection travels up to the pedicle and ultimately the pods are infected severely. The pod showed browning of the placenta and contained abortive seeds with brown discolorations.

3.3 Pathogenicity test

(a) Stab inoculation

The seedlings at staple stage were inoculated, and symptoms appeared within 3 days of inoculation. All the inoculated seedlings showed symptoms within 5 days. The mortality was 96.67% on the 8th day in inoculated seedlings as compared to the control, in which no mortality was observed.

(b) Smoothering of seeds by pure culture of the pathogen

In smoothered seeds, the seed germination was delayed and only radicle emergence in 43.33 and 46.67% of the seeds in samples (A and B) within the 5th day of the Petri plate method as compared to control (100% and 100%) was observed. All the emerged seedlings showed browning and puffing of the radicle followed by rotting and ultimately death of the seedlings. Such symptomatic seedlings were 69.23 and 78.57% of smoothered seeds in samples A and B, respectively, as compared to control. On water agar, the germinated seeds showed rotting followed by the death of seedlings within 15 days of inoculation. It was 100 and 93.33% in control and in smoothered seeds, being 60 and 53.33% in sample A and sample B, respectively. The symptoms on the cotyledonary leaves appeared after 10 days. The symptomatic seedlings were 88.59% and 100% raised from smoothered seeds as compared to control in sample A and sample B, respectively.

The present study is based on the data collected from the Petri plate method, water agar test tube seedling symptoms tests (TTSST), pot experiments, and field observations. Observations showed that the seed infection of *X. axonopodis* pv. *phaseoli* caused poor seed germination with pre-and post-emergence mortality. The loss was more in shrivelled-discolored seeds as compared to asymptomatic seeds. The failure of seed germination and the incidence of seedling mortality were correlated with the degree of discoloration of the seeds.

The seedlings obtained from asymptomatic seeds were more vigorous and had a low incidence of the pathogen and mild symptoms. The pathogen caused typical disease symptoms of brown leaf spots and brown necrotic spots on leaves.

The pathogenicity tests conducted by artificial inoculation of seeds showed similar phytopathological effects and disease symptoms as those shown by naturally infected seeds on sowing.

The discoloured seeds with prominent symptoms in sunflower due to *Pseudomonas syringae* **(**Godika, Agrawal and Singh, 2000**),** pigeon pea seeds (Sharma *et al.* 2002), *X.a.* pv. *vesicatoria* in chilli, *Ralstonia solanacearum* in tomato (Sharma, 2007, Sharma and Sharma, 2014) and brinjal seeds (Sharma, 2016) exhibit water-soaked symptoms (translucent areas). The bacteria have the capacity to colonise germinating seeds, which is an important step in disease transmission.

In a histopathological study, it was observed that bacteria penetrated via funiculus in seed and through stomata in cotyledonary leaves. In soybean, it was found that *Psudomonas syringae* pv. *glycinea* penetrated through stomata and multiplied in intercellular spaces of the mesophyll (Sinclair, 1982, Groth, 1983). The cells of the bacterium Xap potentially occurred at the hilum and micropylar regions in all three seed categories in location studies. The bacteria on the seed surface can cause systemic or vascular infection, and they are often detected in the other seed tissues and seed coat layers (Skoric, 1927, Neergaard, 1977, Singh and Mathur, 2004).

In peas, the raphe of seeds with vascular elements provides a good entry point for *Pseudomonas syringae* (Verma and Agrawal, 2018). The bacteria is found in the inter- and intracellular spaces of soft parenchymatous tissue and causes cell lysis.Under field circumstances, Xap invades the parenchyma tissue and may enter the vasculature. Infestation of *X. a.* pv. *phaseoli* in bean seeds occurs in the micropyle and/or funiculus. In the present investigation, it was found that the bacterium exhibits floral bud and fruit pod. Similar studies were reported due to *Acidovorax citrulli* (which causes bacterial fruit blotch of cucurbits) in the cotyledons of pistil-inoculated seeds and perisperm-endosperm layers (Dutta et al., 2012), *X.a.* pv. *vesicatoria* in chilli (Sharma, 2017), tomato (Sharma, 2007) and pigeon pea (Sharma et al., 2001).

The findings of the study clearly show that Xap seed infection caused poor germination and pre- and post-emergence mortality.The failure of seed germination and seedling mortality were correlated with the degree of discoloration of seeds. The seedlings obtained from non-symptomatic seeds were vigorous with a low incidence of pathogens as compared to discoloured seeds. Infected leaves produced tiny brown spots that coalesced and showed the brightening of leaves. The infected plant bears deformed pods with brown necrotic spots. The infected pods have brown to black discoloured seeds on a brown-coloured placenta.

The pathogenic tests conducted on artificially inoculated seeds showed similar phytopathological effects and disease symptoms as found in naturally infected seeds in the various tests. In *Phaseolous vulgaris* and cotton, inoculation of seeds by *X. c.* pv. *phaseoli* found a significant reduction in seed germination (Valarini and Menten, 1991; Zochowski and Rudolph, 1991). The inoculated leaves showed heavy bacterial growth in the mesophyll cells of cotyledonary leaves. Reddy, Ahmed, and Verma (1986) studied that *Xanthomonas campestris* either killed the seeds before germination or the seedlings soon died after emergence in check pea.

In the present study, the initial symptoms of brown necrotic spots on cotyledonary leaves, hypocotyls, and transition zones of seedlings led to the mortality of seedlings, which increased gradually over time. The pathogen is transmitted through seeds. Gananamanicham and Ward (1982), Ravnikar *et al.* (2001), and Black *et al.* (2001) also reported the development of bacterial spots on seedlings and mature plants showed severe defoliation due to heavy infection of *Xanthomonas* spp. Splitting of hypocotyl, browning of radicle and necrosis of cotyledonary tissues due to infection of *X. campestris* pv. *cajani* in pigeon pea also caused failure of seed germination (Sharma *et al.,* 2002). Transmission of bacterial blight caused by

X. axnopodis pv. *cyamopsidis* in plants of cluster bean was observed in plants raised from naturally infected seeds in the experimental field (Chakravarthy *et al.* 2004, Jain and Agrawal, 2011).

4. CONCLUSIONS

The present study revealed a wide-spread heavy occurrence and incidence of the pathogen in seed samples of black gram. The seed-borne inoculum was found to play a big role in the spread of bacteria from seed to seedling to plant and the development of disease in the crop.

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Table 1: District wise occurrence and percent range of incidence of various seed categories in seed samples of black gram

S. No.	Districts	No. of seeds samples studies	Dry Seed Examination			PTS agar
			I. Asymptomatic seeds	II. Bold-discoloured seeds	III. Shrivelled-discoloured seeds	Incidence
1.	Ajmer	09	09 (42.50-87.50)	09(05.75-52.75)	09(02.75-28.00)	06(47-89)
2.	Alwar	03	03 (65.00-78.00)	03(09.25-32.75)	03(02.25-20.50)	03(56-96)
3.	Bikaner	04	04 (67.75-79.50)	04(09.25-32.75)	04(02.75-19.75)	04(16-97)
4.	Bharatpur	03	03(38.00-80.25)	07(08.50-40.00)	03(07.00-12.25)	06(14-67)
5.	Bhilwara	07	07(45.00-57.50)	04(09.25-28.75)	07(08.25-25.75)	02(31-46)
6.	Bundi	13	13(35.50-80.00)	03(10.50-55.00)	13(11.50-23.50)	11(31-91)
7.	Chittorgarh	04	04(86.50-90.00)	13(06.75-45.25)	04(05.50-09.50)	04(37-93)
8.	Dausa	04	04(71.50-84.50)	04(08.00-18.25)	04(07.25-15.50)	02(19-29)
9.	Dholpur	03	03(35.50-85.50)	03(05.25-36.50)	03(09.25-34.00)	03(32-47)
10.	Jaipur	26	26(45.75-88.25)	26(05.75-45.75)	26(03.25-22.25)	24(20-98)
11.	Jalore	01	01 (62)	01(27)	01(11)	01(22)
12.	Jhunjhunu	05	05(58.50-87.50)	05(07.50-19.75)	05(05.75-21.75)	05(34-96)
13.	Jodhpur	05	05 (62.75-72.25)	05(11.50-17.25)	05(13.50-25.50)	04(47-93)
14.	Karauli	06	06(45.00-90.50)	06(04.50-49.00)	06(05.00-22.25)	05(12-83)
15.	Kota	04	04 (69.00-79.50)	04(05.50-19.75)	04(11.25-24.75)	04(26-89)
16.	Sawai Madhopur	18	18(41.00-86.50)	18(03.15-36.75)	18(03.25-24.50)	16(2-79)
17.	Sriganganagar	04	04(73.75-82.75)	04(04.75-10.5)	04(08.25-21.50)	04(42-93)
18.	Sikar	05	05(63.5-78.25)	05(05.50-19.25)	05(13.50-21.25)	05(42-83)
19.	Sirohi	01	01(85.75)	01(6)	01(08.25)	01(16)
20.	Tonk	33	33(28.25-93.25)	33(02.25-48.50)	33(03.25-36.50)	27(6-100)
21.	Udaipur	15	15(35.50-91.50)	15(04.75-47.75)	15(03.50-27.25)	15(21-92)
	Total	**173**	**173(28.25-93.25)**	**173(02.00-52.75)**	**173(02.25-36.50)**	**152(2-100)**

*The figures in parentheses are the percent incidence range

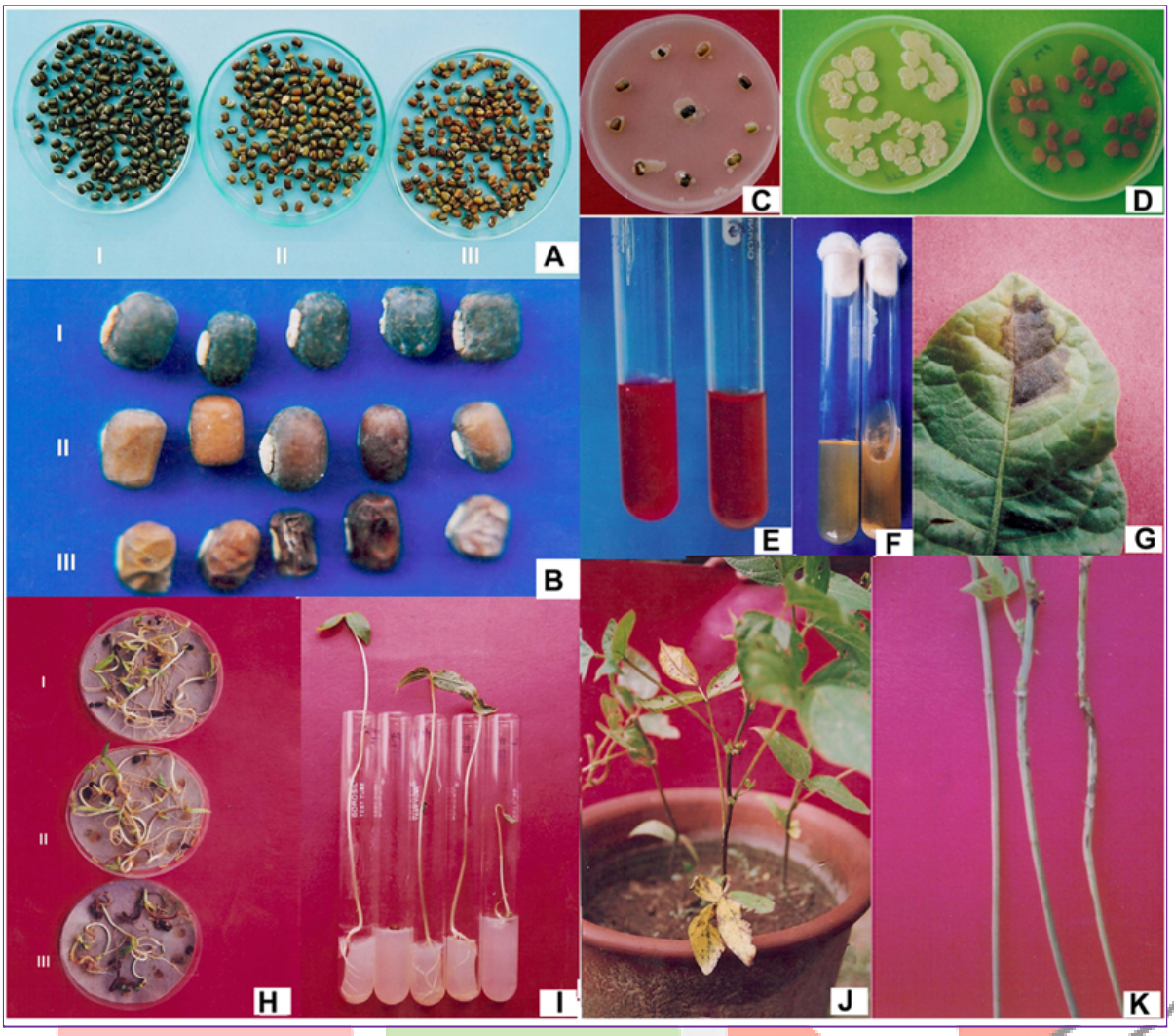
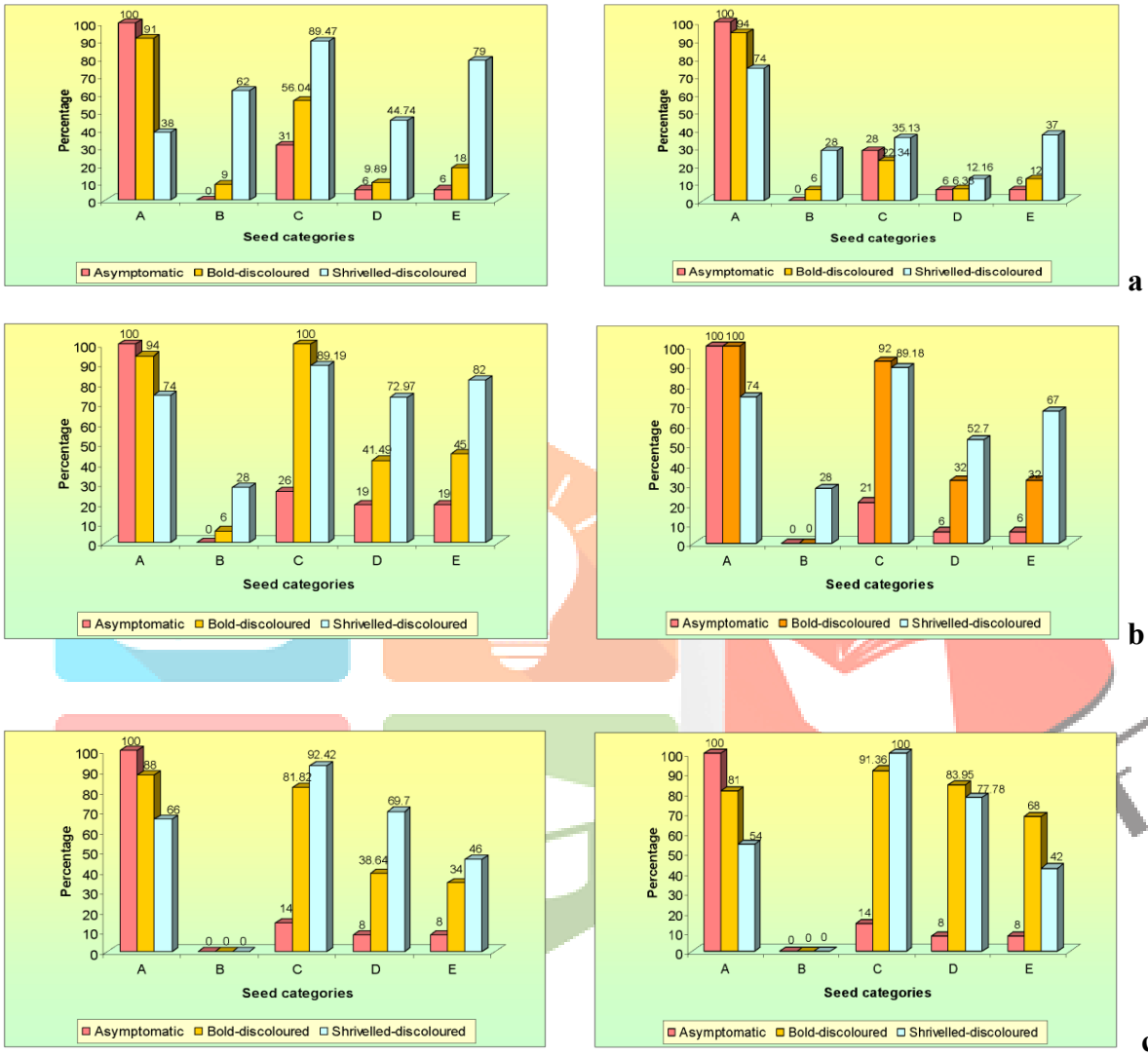


Fig.1. Infection of *Xanthomonas axonopodis* pv. *phaseoli* in seeds of black gram, (A-B) Seed categorized into I asymptomatic, II bold-discolored and III shrivelled-discolored seeds showing degree of discolorations, (C) The characteristic pattern of growth of smooth and entire margin and brown colonies around the seeds on PTS agar medium. (D) Bacterial colonies on PTS medium showing yellow, mucoid colonies with a halo of hydrolysis around the colonies (right). The white colonies without halo were not XPF (left) (E) Arginine dihydrolysis test on Thornley's medium, (F) The liquefaction of gelatin medium, (G) Hypersensitivity reaction (HR) test, (H) Symptoms in seedling in Petri plate method, (I) water agar test tube seedling symptom test, (J) Plant showing disease symptoms of browning and necrosis in pot experiment, (K) Shoots showing bacterial symptoms in brown streaks.

Fig. 2. Phytopathological effect of natural seed infection of *Xanthomonas axonopodis* pv. *phaseoli* in Petri plate method (a), water agar test tube seedling symptoms test (b) and in pot experiment (c).

Sample A (ac. no. Vm – 1104) **Sample B (ac. no. Vm – 1119)**



A– Seed germination; B– Ungerminated seed with pathogen; C– Symptomatic seedling; D– Seedling mortality; E– Total loss