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RESEARCH PAPER

Association of endophytes with soybean crop in northern Karnataka and parts of Maharashtra

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Abstract: A roving survey was undertaken in *kharif* 2017 to collect healthy soybean plants in northern Karnataka *viz.*, Belagavi, Haveri, Bidar, Dharwad and parts of Maharashtra *viz.*, Kolhapur and Sangli districts. A total of 30 fungal (4 from root, 11 from stem and 15 from leaf) and 30 bacterial (6 from root, 13 from stem and 11 from leaf) endophytes were isolated from soybean. Maximum number of endophytes were obtained from Belagavi district (10 fungal and 11 bacterial endophytes) followed by Dharwad district (8 fungal and 7 bacterial endophytes). Maximum endophyte association was observed in irrigated condition (1.38 & 1.63), black soils (1.31 & 1.46) and vegetative stage (1.73 & 1.27). Among the soybean varieties, the highest number of endophytes were isolated from DSb 21 (1.80 & 2.00) followed by KDS 344 (1.00 & 2.00).

Key words: Association, Endophytes, Roving survey, Soybean

Introduction

Soybean (*Glycine max* L. Merrill) is the world's most important seed legume, which contributes to 25 per cent of the global edible oil and about two-thirds of the world's protein concentrate for livestock feeding. Despite having made rapid stride for both coverage and total production, soybean in India still suffers on productivity front. The major constraints in soybean production are climate, rainfall, edaphic factors, biotic and abiotic stresses. Soybean is infected by many fungal, bacterial, viral and nematode diseases. Even though revolutionary development has occurred in pesticide industry with the advent of many new molecules, the risk of pesticide residues, resistance development and environmental safety are still the major concern and has opened the doors for new eco-friendly, sustainable approaches like biological control and integrated disease management.

Endophytes have emerged as a new innovative and sustainable approach to manage the diseases, abiotic stresses and to promote plant growth. Endophyte refers to fungi or bacteria which, for all or part of their lifecycle, invade the tissues of living plants and cause unapparent and asymptotic infections entirely within plant tissues, but cause no symptoms of the disease (Wilson, 1995). Endophytes benefit the plant by promoting plant growth, improving resistance to multiple stress and offering protection from diseases and insects.India being a tropical country with great biodiversity offer more chances to chase the endophytes that are suitable to produce bioactive compounds. The benefits of native endophytes have been recognized over the past ten years from around the world. The great amount of information regarding the key role of endophytic microbes in agriculture is yet to be explored. In India, the information available on soybean endophytes is very less and despite of having diverse microflora, the endophytes still remain as untapped resources. Hence, with a view of isolating the native endophytes in soybean ecosystem and understanding their association under different cropping situations the present investigation was undertaken.

Material and methods

Collection of healthy plant samples

A roving survey was conducted during *kharif*2017 and healthy soybean plants were collected from major soybean growing areas of northern Karnataka *viz.*, Belagavi, Haveri, Bidar, Dharwad and parts of Maharashtra *viz.*, Kolhapur and Sangli districts.Healthy, symptomless soybean plants were uprooted randomly from soybean fields and transported to the laboratory in sterile polythene bags and were used for the isolation of the endophytes within 72 h after sampling.

Isolation of fungal endophytes

Roots, stems and leaves of healthy soybean plants collected during the survey were washed in running tap water to remove soil dirt and debris and were cut into one cm^2 sections. After this, surface sterilization was done with 70 per cent ethanol for a minute followed by one per cent sodium hypochlorite for three minutes. Subsequently, the sections were rinsed with sterile distilled water thrice and placed on nine cm petri plates containing potato dextrose agar (PDA) medium amended with streptomycin sulphate (250 mg l^{-1}) to slow down the bacterial growth. Sterilized tissue segments were pressed onto the surface of PDA medium to check the efficacy of surface sterilization procedure and to confirm endophytic isolations only from internal tissues of the plant segments (Schulz *et al.*,1993). All plates were incubated at 27 ± 1 °C and were observed for fungal growth at three days interval upto two weeks. Fungi growing out from the plant tissues were transferred on to fresh PDA medium. After purifying the isolates several times, final pure cultures were transferred to PDA slants and stored for further studies in refrigerator at 4 °C.

Isolation of bacterial endophytes

Endophytic bacteria from healthy soybean plants were isolated as per the procedure suggested by McInroy and Kloepper (1995). Roots, stems and leaves were washed in running tap water to remove dirt and were split into longitudinal sections. After this, surface sterilization was done

with ethanol (70 %) for a minute followed by sodium hypochlorite (1 %) for three minutes. Subsequently the sections were rinsed with sterile distilled water thrice.Then, the sections were rinsed with 0.02 M potassium phosphate buffer three times (0.1 ml aliquot from the last wash was taken and transferred to Petri plate which served as sterility check). One gram of plant parts was macerated with nine ml of potassium phosphate buffer in pestle and mortar. Further, serial dilution was made up to 10^{-6} dilution. Each dilution was plated onto nutrient agar medium (NA) separately and the endophytic bacterial colonies were observed upto the dilutions of 10^{-3} and not above this dilution. The plates were incubated at 27 ± 1 °C for 48-72 hfor observing the colonies developed on it andisolated colonies were picked up and streaked again on fresh nutrient agar plates, purified and incubated.

Coding of endophytes

Fungal and bacterial endophytes were coded by using four letters. First letter indicated the part of plant (R: Root, S-Stem and L: Leaf) from which endophyte was isolated, second letter indicated the group of endophyte (F- Fungal and B- Bacterial), third letter indicated the district from which endophyte was isolated (B- Belagavi, H- Haveri, B- Bidar, D- Dharwad, K- Kolhapur, S- Sangli) and the last letter indicated the village name (ex. D-Devikoppa, V- Vakkund, S- Sankeshwar).

Results and discussion

Roving survey was undertaken during *kharif* 2017 in major soybean growing areas of northern Karnataka *viz.*, Belagavi, Dharwad, Haveri, Bidar districts and parts of Maharashtra *viz.*, Kolhapur and Sangli districts to collect the apparently healthy soybean plants for the isolation of endophytes (Table1). The survey revealed that maximum number of endophytes were obtained from Belagavi district followed by Dharwad district suggesting the vast variation of microflora in the locality. In Belagavi district, out of seven locations surveyed, a total of 10 fungal and 11 bacterial endophytes were obtained. From Haveri district, a total of four fungal and four bacterial endophytes were obtained out of six locations visited during the survey. In Bidar district, out of four locations surveyed, a total of five fungal and five bacterial endophytes were obtained. In Dharwad district, a total of eight fungal and seven bacterial endophytes were obtained out of six locations visited.In Kolhapur district of Maharashtra, one fungal and two bacterial endophytes were obtained from Kaneriwadi village. A total of two fungal and one bacterial endophyte was obtained from Kasabe Digraj village of Sangli district of Maharashtra. Overall, 30 fungal and 30 bacterial endophytes were obtained from 25 different locations of northern Karnataka and parts of Maharashtra.

Association of endophytes in soybean under various crop conditions

The association of endophytes varied with different crop conditions *viz.*, crop situation, soil types, crop growth stages and varieties of soybean grown. The data are presented in Table 2.

The results revealed that the association of endophytes was maximum under irrigated condition with a mean endophyte isolation of 1.38 and 1.63 for fungal and bacterial endophytes, respectively. Under rainfed condition, even though higher number of endophytes were obtained, the association was minimum with a mean endophyte isolation of 1.12 and 1.00 for fungal and bacterial endophytes, respectively. This could be due to the presence of sufficient moisture, good biomass of the crop in irrigated condition which supplied nutrients and enhanced the endophyte survival and diversity.

The data on survey and isolation of endophytes from different soils showed that the association of endophytes was maximum under black soil with a mean endophyte isolation of 1.31 and 1.46 for fungal and bacterial endophytes, respectively. In red soils, the mean endophyte isolation was 1.08 and 0.92 for fungal and bacterial endophytes, respectively which accounted for minimum endophyte association.It may be due the high moisture holding capacity and fertility of black soils which supported the microbial growth.

The association of endophytes was maximum at vegetative stage with a mean endophyte isolation of 1.73 and 1.27 for fungal and bacterial endophytes, respectively when compared with flowering and pod filling stage and revealed that the number of endophytes obtained decreased with the increase in the age of the crop. It could have been due to lack of essential nutrients at later stages of crop growth. Similar results were observed by McInroy and Kloeper (1995) and Pimental *et al.* (2006) who showed that some of the essential nutrients needed by endophytic bacteria were unavailable during the maturation and senescence of plants. The association of endophytes was maximum in DSb 21 variety with maximum mean fungal and bacterial isolation of 1.80 and 2.00, respectively followed by KDS 344 variety with mean fungal and bacterial isolation of 1.00 and 2.00, respectively. The varieties JS 335 and JS 93-05 showed minimum endophyte association. It may be due to the fact that DSb 21 being rust resistant variety offers less biotic stress environment inside the plant and allows for the good colonization of the endophytes as they are less affected by the pathogens.

Fungal endophytes

A total of 30 fungal (4 from root, 11 from stem and 15 from leaf) and endophytic isolates were obtained from different parts of healthy soybean samples which were collected from 25 locations in four districts of northern Karnataka and two districts of Maharashtra.Similarly, Aharwal *et al*. (2014) and Meenatchi *et al*. (2016) reported that the colonization rate in the leaves was foundto be significantly higher than other parts of the plant. Cultural characters of the fungal endophytes were recorded on PDA. Colony characteristics including colour, margin, mycelial growth, texture and colony diameter were recorded after ten days of incubation (Table 3). Among the 30 fungal endophytes, the colony colour varied from white to black, margin from regular to irregular, smooth to coarse texture and flat to raised mycelial growth. The colony diameter varied from 35 mm to 90 mm but maximum number of isolates showed 90 mm colony diameter after ten days of incubation.

Table 1. Survey for collection and isolation of endophytes from apparently healthy soybean plants in northern Karnataka and parts of Maharashtra during *kharif* 2017

State	District	Taluka	Village	GPS		Soil type	RF/IR	Stage of the crop	Variety	Diseases noticed	No. of endophytes obtained	
				Lat (°N)	Long (°E)						F	B
Karnataka	Belagavi	Chikkodi	Chikkodi	16.25	74.35	Red	RF	Flowering	JS 335	ALS, CLS	1	-
			Jainapur	16.28	74.40	Red	RF	Vegetative	JS 93-05	ALS	2	2
		Athani	Ugarkhurd	16.38	75.15	Black	IR	Pod filling	DSb 21	Anthracnose	1	3
			Koligudda	16.30	75.10	Black	RF	Vegetative	JS 93-05	ALS	1	1
		Hukkeri	Sankeshawar	16.16	74.28	Red	RF	Vegetative	JS 335	CLS, Rust	1	1
			Khanapur	15.56	74.50	Black	IR	Pod filling	JS 335	Anthracnose	-	1
		Bailhongal	Vakkund	15.81	74.85	Black	IR	Vegetative	DSb 21	-	4	3
										Total	10	11
	Haveri	Shiggavi	Shiggavi	14.99	75.22	Red	RF	Vegetative	JS 93-05	-	1	1
			Hanumarahalli	14.58	75.28	Red	RF	Vegetative	JS 335	Anthracnose	1	-
		Savanur	Basavanakoppa	14.97	75.34	Red	RF	Pod filling	JS 93-05	CLS	-	-
		Hangal	Akkialur	14.76	75.13	Black	IR	Flowering	JS 335	Rust	-	1
			Balambeda	14.75	75.11	Black	IR	Flowering	JS 335	Rust	1	1
		Byadgi	Yadagundi	14.68	75.38	Black	IR	Flowering	JS 335	CLS	1	1
										Total	4	4
	Bidar	Bidar	Janawada	17.51	77.50	Black	RF	Flowering	JS 335	Rust	1	1
		Bhalki	Bhatambra	18.06	77.16	Black	RF	Vegetative	JS 335	CLS, ALS	2	1
		Humnabad	Itagi	17.79	77.15	Red	RF	Flowering	JS 93-05	ALS	1	2
		Basavakalyan	Narayanpur	17.86	76.94	Black	RF	Flowering	JS 93-05	Anthracnose	1	‘1
										Total	5	5
	Dharwad	Dharwad	MARS, Dharwad	15.45	75.00	Black	IR	Vegetative	DSb 21	ALS, CLS	2	2
			Narendra	15.36	75.12	Red	RF	Pod filling	JS 93-05	ALS, wilt	1	1
			Garag	15.52	74.49	Red	RF	Pod filling	DSb 21	Wilt	1	1
		Hubballi	Shirguppi	15.35	75.28	Red	RF	Vegetative	JS 335	Rust and ALS	2	-
		Kalagatagi	Kalagatagi	15.14	75.17	Red	RF	Vegetative	JS 335	Rust, CLS	1	2
			Devikoppa	15.15	75.14	Red	RF	Pod filling	DSb 21	Wilt	1	1
										Total	8	7
Maharashtra	Kolhapur	Karvir	Kaneriwadi	16.57	74.12	Black	RF	Flowering	KDS 344	ALS, CLS	1	2
	Sangli	Miraj	KasabeDigraj	16.91	74.51	Black	IR	Vegetative	JS 335	Anthracnose	2	1
										Total	3	3
										Grand total	30	30

GPS- Global Positioning System, Lat- latitude, Long- longitude, RF- Rainfed, IR- Irrigated, F- Fungi, B- Bacteria, ALS- Alternaria leaf spot, CLS- Cercospora leaf spot

Table 2. Association of endophytes in soybean under various crop conditions

Particulars		Number of		Mean fungal endophyte isolation	No. of bacterial endophytes isolated	Mean bacterial endophyte isolation
		Fields visited	Fungal endophytes isolated			
Crop situation	Rainfed	17	19	1.12	17	1.00
	Irrigated	08	11	1.38	13	1.63
Soil types	Red	12	13	1.08	11	0.92
	Black	13	17	1.31	19	1.46
Crop growth stages	Vegetative	11	19	1.73	14	1.27
	Flowering	08	07	0.88	09	1.13
	Pod filling	06	04	0.67	07	1.17
Varieties	DSb 21	05	09	1.80	10	2.00
	JS 335	12	13	1.08	10	0.83
	JS 93-05	07	07	1.00	08	1.14
	KDS 344	01	01	1.00	02	2.00

Table 3. Cultural characters of fungal endophytes isolated from major soybeangrowing areas of northern Karnataka and parts of Maharashtra during *kharif* 2017

Source of isolation	District	Location	Fungal endophytecode	Colony colour	Margin	Texture	Mycelial growth	Colony diameter after 10 days (mm)
Root	Belagavi	Chikkodi	RF-BC-1	White	Regular	Smooth	Flat	85
		Khanapur	RF-BKh-2	Grey	Regular	Coarse	Flat	85
		Vakkund	RF-BV-3	Grey	Irregular	Coarse	Raised	85
	Dharwad	Kalagatagi	RF-DK-4	Light grey	Irregular	Coarse	Raised	85
Stem	Belagavi	Ugarkhurd	SF-BU-1	Black	Regular	Smooth	Flat	35
		Koligudda	SF-BK-2	Light grey	Irregular	Coarse	Raised	85
		Vakkund	SF-BV-3	Dull white	Regular	Smooth	Flat	85
		Vakkund	SF-BV-4	White	Regular	Smooth	Flat	83
	Haveri	Shiggavi	SF-HS-5	Grey	Regular	Smooth	Flat	40
		Balambeda	SF-HB-6	Black	Regular	Smooth	Flat	50
	Bidar	Bhatambra	SF-BiB-7	White	Regular	Smooth	Flat	85
	Dharwad	MARS, Dharwad	SF-DM-8	Grey	Irregular	Coarse	Raised	85
		Narendra	SF-DN-9	Light grey	Regular	Coarse	Flat	85
		Shirguppi	SF-DS-10	Light grey	Irregular	Coarse	Raised	85
	Sangli	Kasabe Digraj	SF-SK-11	Light grey	Irregular	Coarse	Raised	85
Leaf	Belagavi	Jainapur	LF-BJ-1	Light grey	Regular	Smooth	Raised	85
		Jainapur	LF-BJ-2	Grey	Irregular	Smooth	Raised	83
		Sankeshwar	LF-BS-3	Dull white	Irregular	Smooth	Flat	55
		Vakkund	LF-BV-4	White	Regular	Smooth	Raised	63
	Haveri	Hanumarahalli	LF-HH-5	Light grey	Regular	Coarse	Raised	85
		Yadagundi	LF-HY-6	White	Regular	Smooth	Raised	85
	Bidar	Bhatambra	LF-BiB-7	Light grey	Irregular	Coarse	Flat	85
		Itagi	LF-BiI-8	Grey	Regular	Smooth	Raised	83
		Narayanpur	LF-BiN-9	Dull white	Regular	Smooth	Flat	85
	Dharwad	MARS, Dharwad	LF-DM-10	Light grey	Regular	Coarse	Flat	85
		Garag	LF-DG-11	Dull white	Irregular	Smooth	Flat	55
		Shirguppi	LF-DS-12	White	Irregular	Smooth	Raised	53
		Devikoppa	LF-DD-13	Light grey	Irregular	Smooth	Flat	58
	Kolhapur	Kaneriwdai	LF-KK-14	White	Regular	Smooth	Raised	84
	Sangli	Kasabe Digraj	LF-SK-15	White	Regular	Smooth	Flat	85

RF- Root fungal endophyte, SF- Stem fungal endophyte, LF- Leaf fungal endophyte, B- Belagavi, H- Haveri, Bi- Bidar, D- Dharwad, K- Kolhapur, S- Sangli

Bacterial endophytes

A total of 30 bacterial endophytes were isolated (root- 6, stem-13 and leaf-11) from different parts of healthy soybean samples which were collected from 25 different locations in four districts of northern Karnataka and two districts of Maharashtra. The growth of isolates on nutrient agar medium showed considerable differenceswith respect to colony colour, appearance (form), elevation and margin (Table 4). Colony colour varied from white to yellow, circular to irregular form, flat to raised elevation and undulated to entire margin. The Gram

Table 4. Cultural characters of bacterial endophytesisolated from major soybeangrowing areas of northern Karnataka and parts of Maharashtra during *kharif* 2017

Source of isolation	District	Location	Bacterial Endophyte code	Colony colour	Colony form	Elevation	Margin	Gram reaction
Root	Haveri	Shiggavi	RB-HS-1	Light Yellow	Circular	Raised	Entire	-
		Balambeda	RB-HB-2	White	Circular	Convex	Entire	+
		Yadagundi	RB-HY-3	Yellow	Irregular	Convex	Undulated	-
	Dharwad	MARS, Dharwad	RB-DM-4	Yellow	Circular	Raised	Entire	-
		Kalagatagi	RB-DK-5	White	Irregular	Flat	Undulated	+
	Kolhapur	Kaneriwadi	RB-KK-6	Light yellow	Circular	Raised	Entire	-
Stem	Belagavi	Jainapur	SB-BJ-1	Light yellow	Circular	Flat	Entire	-
		Jainapur	SB-BJ-2	White	Irregular	Raised	Undulated	+
		Ugarkhurd	SB-BU-3	Yellow	Irregular	Convex	Undulated	-
		Ugarkhurd	SB-BU-4	Light yellow	Circular	Convex	Entire	-
		Koligudda	SB-BK-5	Yellow	Circular	Raised	Entire	-
		Sankeshwar	SB-BS-6	White	Irregular	Raised	Undulated	+
		Khanapur	SB-BKh-7	Yellow	Circular	Convex	Entire	-
		Vakkund	SB-BV-8	White	Circular	Raised	Entire	-
	Bidar	Janawada	SB-BiJ-9	Yellow	Circular	Raised	Entire	-
	Dharwad	MARS, Dharwad	SB-DM-10	Yellow	Circular	Flat	Entire	-
		Garag	SB-DG-11	Yellow	Circular	Convex	Entire	-
		Kalagatagi	SB-DK-12	Yellow	Irregular	Flat	Undulated	-
		Devikoppa	SB-DD-13	White	Irregular	Raised	Undulated	+
Leaf	Belagavi	Ugarkhurd	LB-BU-1	White	Irregular	Flat	Undulated	+
		Vakkund	LB-BV-2	White	Irregular	Flat	Undulated	+
		Vakkund	LB-BV-3	Yellow	Irregular	Raised	Entire	-
	Haveri	Akkialur	LB-HA-4	Light yellow	Circular	Raised	Undulated	+
	Bidar	Bhatambra	LB-BiB-5	Yellow	Circular	Convex	Entire	-
		Itagi	LB-BiI-6	Yellow	Circular	Convex	Entire	-
		Itagi	LB- BiI -7	White	Irregular	Flat	Undulated	+
		Narayanpur	LB- BiN -8	White	Irregular	Flat	Undulated	+
	Dharwad	Narendra	LB-DN-9	Light yellow	Circular	Raised	Entire	+
	Kolhapur	Kaneriwadi	LB-KK-10	Yellow	Circular	Convex	Entire	-
	Sangli	KasabeDigraj	LB-SK -11	Yellow	Circular	Convex	Entire	-

RB- Root bacterial endophyte,SB- Stem bacterial endophyte, LB- Leaf bacterial endophyte, Gram reaction: +: Gram positive
-: Gram negative, B- Belagavi, H- Haveri, Bi- Bidar, D- Dharwad, K- Kolhapur, S- Sangli

reaction for bacterial endophytes revealed that maximum number of isolates were gram positive (19) and 11 were gram negative. Colony morphology gave an indication of the variation among the endophytes. Similar observations were made by Zinniel *et al*. (2002) and Hung and Annapurna (2004).

Conclusion

A total of 30 fungal and 30 bacterial endophytes were obtained from major soybean growing areas of northern Karnataka *viz.*, Belagavi, Dharwad, Haveri, Bidar districts and parts of Maharashtra *viz.*, Kolhapur and Sangli districts. Maximum number of endophytes were obtained from Belagavi district followed by Dharwad district. Maximum association of endophytes were observed in irrigated condition, black soils and vegetative stage. Highest number of endophytes were isolated from DSb 21 followed by KDS 344 varieties of soybean. This is the first report on endophytes and their association with soybean ecosystem from Karnataka.

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