International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(5): 2128-2133

Received: 22-07-2020
Accepted: 24-08-2020

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Identification of chilli (*Capsicum Annuum* L.) cultivars based on chemical tests and SDS-page of total soluble proteins

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DOI: https://doi.org/10.22271/chemi.2020.v8.i5ac.10622

Abstract
Identification of chilli cultivar is prime importance to ensuring seed quality during seed production, certification and quality control. Fifteen cultivars were subjected to different biochemical tests *Viz*., phenol test, modified phenol test with $FeSO_4$ and $CuSO_4$, analysis of soluble seed proteins by SDS-PAGE. No single varieties were so distinct in case of phenol colour reaction, while it helped to group the cultivars. Based on modified phenol text with ferrous sulphates 1per cent ($FeSO_4$) and 0.5 per cent copper sulphate ($CuSO_4$) helped in further classification of cultivars. The cultivars Gouribidanur Local and Phule Jyothi showed unique bands at 14 (Rm: 0.624) and 2 (0.191) level of band. All the cultivars were distinct among each other showing SDS-PAGE as efficient method in chilli cultivar identification.

Keywords: Characterization, phenol test, SDS-page

Introduction
India is the largest producer of chilli in the world accounting nearly 90 per cent of world cultivation (Anon., 2003c). The area under cultivation during 2004-5005 was 0.94 million hectares with a production of 1.03 million tones. Chilli occupied the top slot in the list of spices exported from India during 1995-96 surpassing the traditional item black pepper and the export of chilli has reached a record level of 55,750 tons valued at Rs. 210.3 million (Gosh *et al*., 1997). In spite, the importance of local cultivars, which are selected and domesticated over a period of time, generations by the farmers for their adaptation to different climatic conditions, preferences in quality, biotic and abiotic stresses, cultural values, medicinal properties and yield are to be emphasized. Out of which many cultivars are now in seed production chain. However, there is lack of compiled key diagnostic characters of these cultivars, which is essential to carry out scientific seed production, enforce proper quality control and to promote seed trade. Rouging and the ODV test are effective tools to maintain genetic purity for which discerned specific and stable characters are the need of the hour.
In order to maintain genuineness and quality of seed, seed technologists must be well equipped to identify different cultivars at seed level. Varietal description given by the breeder most often relates to field characters and insufficient to identify seed lot of a cultivar effectively. In the changing global scenario of the post-GATT era, the government of India has enacted Plant Varieties Protection Act and Farmer Rights Act, 2001, which provide protection to new, extinct varieties and germplasm. To qualify for protection under this act, the variety must be evaluated for its DUS (Distinctness, Uniformity and Stability) and VCU (Value for cultivation and use) tests. Morphological descriptors have traditional significance and have been adopted as classical taxonomic approach for identification of crop varieties. However, this is time consuming, requires large areas and highly skilled personnel, making subjective decisions due to variations in environmental reactions. Further, many of the morphological descriptors used are mutagenic, quantitative or continuous characters, the expression of which are altered by environmental factors and the analysis of which requires statistical tests. These are compelling reasons to find more rapid and cost-effective procedures that could augment morphologically based approaches that are directly applicable to seeds. Such as biochemical reaction of seeds to phenol and electrophoretic analyses of soluble seed proteins are the need of the moment.

Material and Methods

Fifteen chilli cultivars were subjected to different biochemical and chemical tests for characterization at electrophoresis lab, Seed Technology Research unit, NSP, Bangalore.

Standard and modified phenol test

Two hundred (50 x 4) seeds were presoaked in distilled water for 24 h at 25 ± 1°C, they were transferred on to two layers of Whatman No.1 filter paper saturated with one per cent phenol solution. For modified phenol test with Ferrous Sulphate (0.5% $FeSO_4$) and Modified phenol test with Copper Sulphate (0.5 % $CuSO_4$) were used. Then the petri dishes were covered and incubated at 25 ± 1°C. The colour reactions were noted after 24 h based on the colour development of seed coat. The selected cultivars were classified into four categories *viz*., No change in colour, Very Light brown and Light Brown (Jaiswal and Agarwal, 1995) [10].

Electrophoretic analyses of soluble seed proteins

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) of total soluble seed proteins was carried out by using 12 per cent gels as per Laemmli (1970). Protein was extracted from three seed by adding 0.2 ml Tris-HCI extraction buffer (25 mM, pH 8.3) and suspension was centrifuged at 10,000 rpm for 10 minutes, extract was dissolved in equal amount of working buffer (Tris-HCI 0.0625 M, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 15% glycerol and 0.001% bromophenol blue) and kept in boiling water for 5 minutes, again centrifuged and the supernatant was used for loading. A current of 1.5 mA per well with a voltage of 80 V was applied until the tracking dye crossed the stacking gel. Later the current was increased to 2 mA per well and voltage up to 120 V. The electrophoresis was stopped when the tracking dye reached the bottom of the resolving gel. Then the gel was stained overnight with coomaasie brilliant blue solution and destained using a mixture of 227 ml of methanol, 46 ml of acetic acid and 227 ml of distilled water until the bands were clearly visible.

Results and Discussion

Phenol colour reaction depends on the quality of oxidative enzymes and monophenol oxidase present in seeds. Phenol colour reaction, is an index of polyphenol oxidase activity, has been utilized to distinguish the crop varieties (Joshi and Banerjee, 1977; Abrol and Uperty, 1972 [1]; Chauhan and Nanda, 1989; Sparks *et al*., 1985) [15]. In the present study, fifteen cultivars were characterized (Table1.) using reaction to standard phenol and modified phenol ($FeSO_4$/$CuSO_4$).

Standard phenol test

This test discriminated the cultivars in to groups *viz*., no colour change, very light brown, light brown and brown (*Fig*.2). Attigeri local, Byadagi Dabba, Byadagi Kaddi, Koira local and Kuchangi Local I did not respond to the standard phenol reaction. All other cultivars responded and were grouped depending upon the intensity of staining.

Modified phenol test

Modified phenol test (1% $FeSO_4$) helped in further classification of cultivars (*Fig*.3). Kuchangi Local I was found unique from all other cultivars by its no colour change, Byadagi Dabba and Attigeri Local were also distinct by their light brown colour. All other cultivars were grouped under either brown, dark brown or black. Modified phenol test with 0.5 per cent $CuS0_4$ was also found to distinguish cultivars. Koira Local and Attigeri Local were distinct by their no colour change, where as all other cultivars were grouped either into light brown, brown or dark brown (Table 1). The presence of metallic ions Fe^{++} and Cu^{++} in modified phenol test was found to enhance phenol colour reaction since, it is an enzymatic reaction and these ions acts as catalyst (Banerjee and Chandra, 1977), which was further confirmed by Gupta and Agrawal (1988) [8], Agarwal and Karki (1989) [2]. Phenol colour reaction of seeds is due to the differences in the genetic background, presumably concerning the enzyme system. (Takshashi, 1983) [16] which can be employed for characterization and identification of varieties.

Keys developed using Standard Phenol and Modified phenol test with 0.5 per cent $CUS0_4$ and 1per cent $FeSO_4$ test showed distinct characteristic reaction patterns and identified all the fifteen varieties (Fig.1).

Protein polymorphism of seed

An attempt to reveal the variation in the total soluble seed proteins of 15 different cultivars was made in the pattern of protein profiles (*Fig*.4 and 5). The cultivars differed with respect to number of bands, their relative mobility on the gel and intensity (Table 2 &3). By SDS-PAGE the total soluble seed proteins could be fractionated into 17 (1 o 8 cultivars) and 16 bands (9 to15 cultivars), which showed heterogeneity among different cultivars studied. A maximum of 13 bands was observed in Gouribidanur Local and was least in Koira Local (5 bands). All the cultivars can be characterized based on number and intensity of band and their relative mobility. The cultivars Gouribidanur Local and Phule Jyothi showed two unique bands at 14 (Rm: 0.624) and 2 (Rm: 0.191) level of band. These two cultivars can be clearly identified by the presence of their unique band.

In region A, cultivars 1-8 only Gouribidanur Local and Chikkaballapur Local showed presence of band and no band was found in cultivars 9-15. Region 'B' could not able to differentiate any of the cultivars. However, Region 'C' found to be very effective in characterization of all 15 studied cultivars of pepper. In this region maximum of 7 bands appeared in cultivars 1-8 and eight bands in cultivars 9-15. Maximum of seven bands were shown by Ballikai, Byadagi Dabba, Phule Jyothi and least (1) in Koira local. Band 3 (Rm: 0.187) cultivars 1-9 was present in Ballikai, Byadagi Dabba, Chikkaballapur Local, Gouribidanur Local and could be used to identify these cultivars. Annigeri local. Attigeri Local, Ballikai and Byadagi Dabba were distinct from other cultivars by presence of band 4 (Rm: 0.218) and band 5 (Rm: 0.242). Even though in this region cultivar specific bands were absent, all the profiles were unique to each cultivar which allowed the identification with each other. Band 3 (9-15) was found only in Phule Jyothi and Tiwari, with light intensity, band 5 (Rm: 0.320) and band 6 (Rm: 0.351) were found in Minchalli Betta, Phule Jyothi, Tiwari and Utkal Awa. These cultivars can be differentiated by other cultivars through presence of these bands. Band 7 (Rm: 0.382) and band 9 (0.462) were absent only in Koira Local and present in other the cultivars and this absence could be used as negative marker to identify this cultivar.

In Region D only two bands appeared both in cultivars 1-8 and 9-15. In cultivars 1-8, band l0 is monomorphic indicating species specificity. Light intense band II (Rm: 0.421) was absent only in Byadagi Dabba and Chittara Chamba hence it can also be used as negative marker to identify these cultivars. In cultivars 9-15, Minchalli Betta, Tiwari Utkal Awa showed light intense band 11 (Rm: 0.629), hence this band could be

used as marker to identify these varietieIn region E, cultivars 1-8, band 13 (Rm: 0.624) and band 15 (Rm: 0.818) were monomorphic in all the cultivars. However, band 14 (Rm: 0.819) was unique to cultivar Gouribidanur Local. Similarly in case of 9-15 cultivars, band II (Rm: 0.629) was found to monomorphic. Light intense band 13 (Rm: 0.771) was unique to Kuchangi Local II and the same may be utilized for identification of Kuchangi local II efficiently which had similar morphological traits as that of Kuchangi Local I and difficult to differentiate at field level. This region could able to produce maximum of two unique bands.

In region F, in cultivars 1-8, all the cultivars showed monomorphic bands. However, 9-15 cultivars showed dark intensity monomorphic band 15 (Rm: 0.932) and another band with (Rm: 0.960) was absent only in cultivars Koira Local and Kuchangi Local II, Such differences in protein polymorphism among cultivarare also noticed by Anderson and Mc Daniel (1979) [4], Odeigah *et al*., (1999) [13], Lucchese (1999) [12], in rice, Chakraborti (1992) [6], Breto (1993) [5] in. tomato and Hasan and Isa, (1998) [9] in Brinjal.

Every cultivar showed unique protein profile and aided in identifying them individually and distinctly. Such identification was also strongly supported by (Lucchese *et al*., (1990) in pepper, tomato and Ahokas, (2002) [3] in barley, oat, wheat, peas and turnip. Analysis of soluble seed proteins from seed provide a reliable key for cultivar Characterization and can be effectively used for distinction and identification. As the expression of protein is unaffected by environmental interaction and can be conveniently used as biochemical marker in identifying Cultivars (Cooke, 1987).

Table 1: Varietal identification of chilli pepper cultivars by Standard phenol test and modified phenol test

Sl. No.	Cultivars	Standard Phenol Test	Modified Phenol Test	
			$CuSo_4$ (0.5 %)	$FeSo_4$ (1 %)
1	Annigeri Local	BR	BL	BR
2	Attigeri Local	NC	LB	NC
3	Ballikai	LB	BL	DB
4	Byadagi Dabba	NC	LB	LB
5	Byadagi Kaddi	NC	BR	LB
6	Chittara Chamba	LB	DB	LB
7	Chikkaballapur Local	LB	DB	LB
8	Gouribidanur Local	VLB	DB	BR
9	Koira Local	NC	BR	NC
10	Kuchangi Local I	NC	NC	LB
11	Kuchangi Local II	VLB	BR	BR
12	Minchalli Betta	BR	BL	DB
13	Phule Jyothi	BR	DB	DB
14	Tiwari	BR	BL	DB
15	Utkal Awa	VLB	DB	BR

Note:

NC – No colour change VLB – Very Light Brown LB – Light Brown

BR – Brown DB – Dark Brown BL – Black

Table 2: Number of total soluble seed protein bands observed in chilli Cultivars

Sl. No.	Cultivars	Low intensity	Medium intensity	High intensity	Total
1.	Annigeri Local	5	4	4	13
2.	Attigeri Local	8	1	4	13
3.	Ballikai	5	6	4	15
4.	Byadagi Dabba	5	6	4	15
5.	Byadagi Kaddi	5	-	4	9
6.	Chittara Chamba	5	-	4	9
7.	Chikkaballapur Local	9	-	4	13
8.	Gouribidanur Local	9	1	4	14
9.	Koira Local	1	-	3	4
10.	Kuchangi Local I	3	1	2	6
11.	Kuchangi Local II	5	2	2	9
12.	Minchalli Betta	7	3	3	13
13.	Phule Jyothi	8	3	3	14
14.	Tiwari	11	-	3	14
15.	Utkal Awa	7	3	3	13
	Total	93	30	51	174

Table 3: Intensity and relative mobility of total soluble seed proteins of chilli Cultivars

Band No.	Region	Rm Value	Annigeri Local	Attigeri Local	Ballikai	Byadagi Dabba	Byadagi. Kaddi	Chittara. Chamba	CKBP	Gouribidanur Local
1	A	0.078	-	-	-	-	-	-	+	+
2	B	0.133	-	-	+	+	-	-	+	+
3	C	0.187	-	-	+	+	-	-	+	+
4		0.218	+	+	++	++	-	-	-	-
5		0.242	+	+	++	++	-	-	-	-
6		0.260	+	+	++	++	+	+	+	+
7		0.296	++	+	+	+	-	-	-	-

8		0.309	++	+	++	++	+	+	+	+
9		0.327	++	+	++	++	+	+	+	+
10	D	0.363	+	+	+	+	+	+	+	+
11		0.412	+	+	+	+	-	-	-	+
12	E	0.484	++	++	++	++	+	+	+	++
13	F	0.557	+++	+++	+++	+++	+++	+++	+++	+++
14		0.624	-	-	-	-	-	-	-	+
15		0.751	+++	+++	+++	+++	+++	+++	+++	+++
16	G	0.818	+++	+++	+++	+++	+++	+++	+++	+++
17		0.854	+++	+++	+++	+++	+++	+++	+++	+++

Note. - - Absent; + - Low intensity; ++ - Medium intensity; +++ - High intensity

Table 4: Intensity and relative mobility of total soluble seed protein of chilli Cultivars. (Contnued…)

Band No.	Region	Rm Value	Koira Local	Kuchangi local I	Kuchangi local II	Minchalli Betta	Phule Jyothi	Tiwari	Utkal Awa
-	A	-	-	-	-	-	-	-	-
1.	B	0.129	-	-	-	+	+	+	+
2.	C	0.191	-	-	-	-	+	-	-
3.		0.240	-	-	-	-	+	+	-
4.		0.271	-	-	-	-	+	+	+
5.		0.320	-	-	-	+	+	+	+
6.		0.351	-	-	-	+	+	+	+
7.		0.382	-	+	++	++	++	-	+
8.		0.419	+	+	+	++	++	+	++
9.		0.462	-	+	+	++	++	+	++
10.	D	0.524	-	-	+	+	+	+	+
11.		0.629	-	-	-	+	-	+	+
-	E	-	-	-	-	-	-	-	-
12.	F	0.709	+++	+++	+++	+++	+++	+++	+++
13.		0.771	-	-	+	+	+	+	+
14.		0.864	-	-	+	-	-	-	-
15.	G	0.932	+++	++	++	+++	+++	+++	+++
16.		0.960	+++	+++	+++	+++	+++	+++	+++

Note. - - Absent; + - Low intensity; ++ - Medium intensity; +++ - High intensity

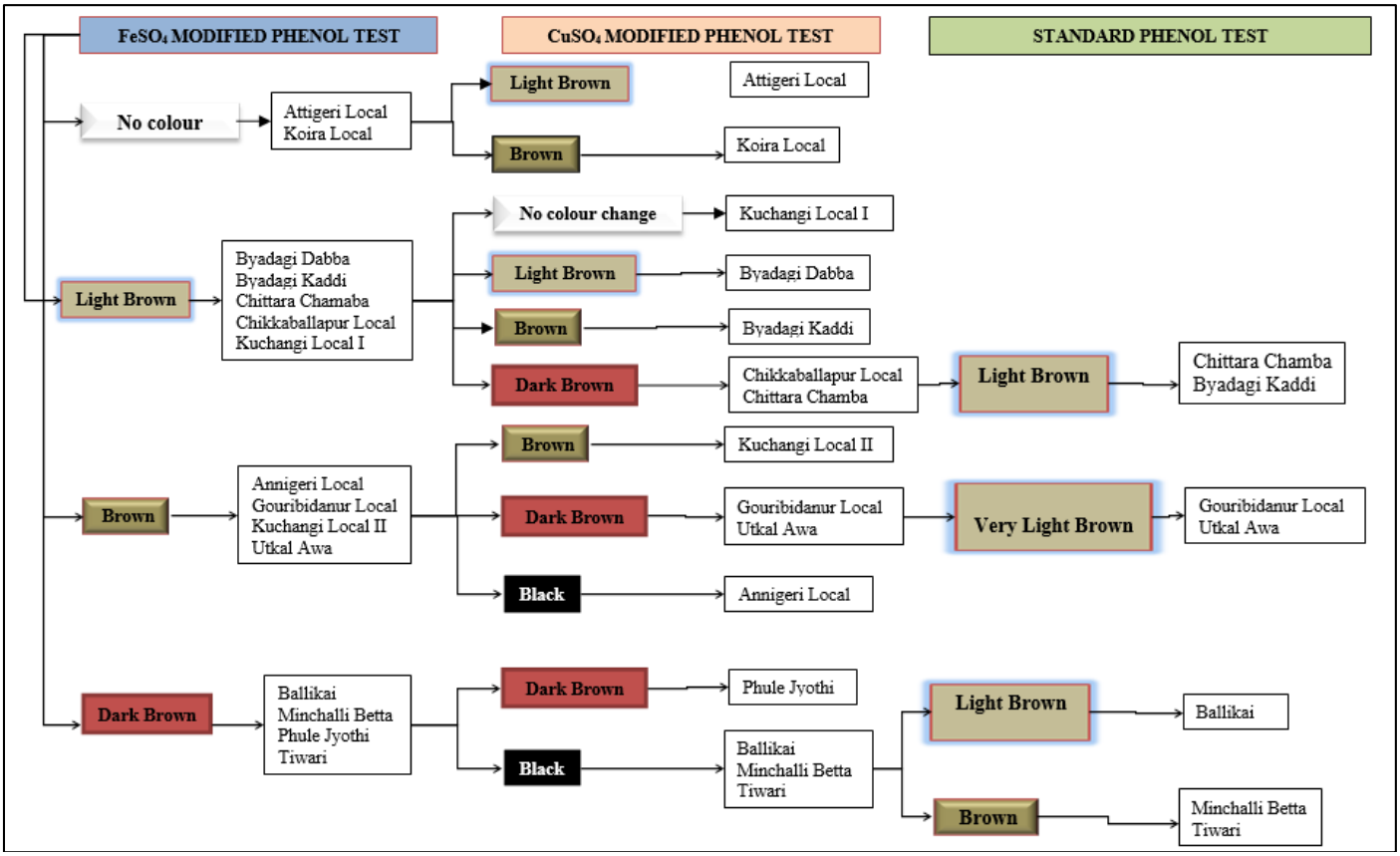

Fig 1: Key for identification of chilli cultivars based on phenol biochemical tests

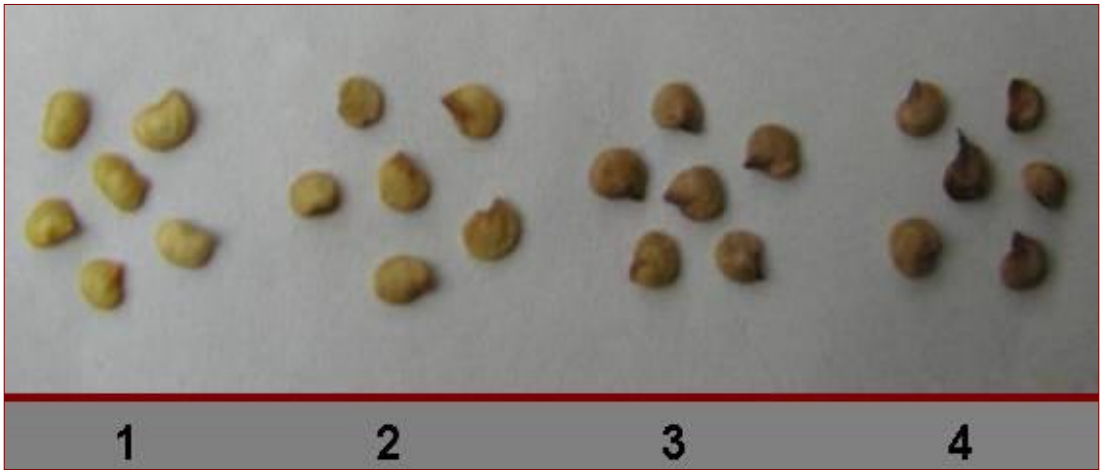

Fig 2: Response of cultivars to standard phenol test. 1. No change 2. Very Light brown 3. Light Brown 4. Brown

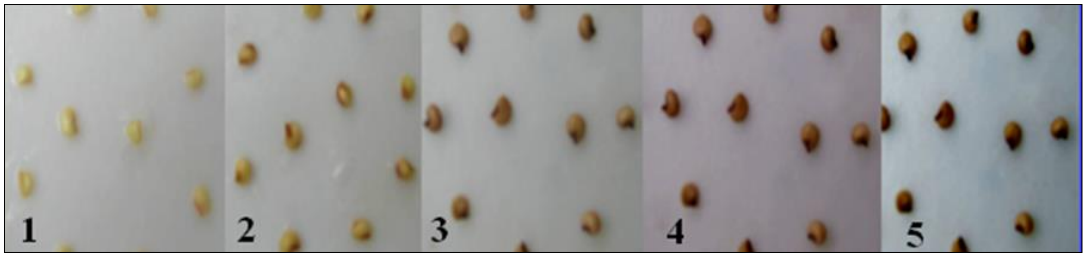

Fig 3: Response of different cultivars to modified phenol test with 0.5 (%) $FeSO_4$. **1.** No change **2.** Light Brown **3.** Brown **4.** Dark Brown **5.** Black

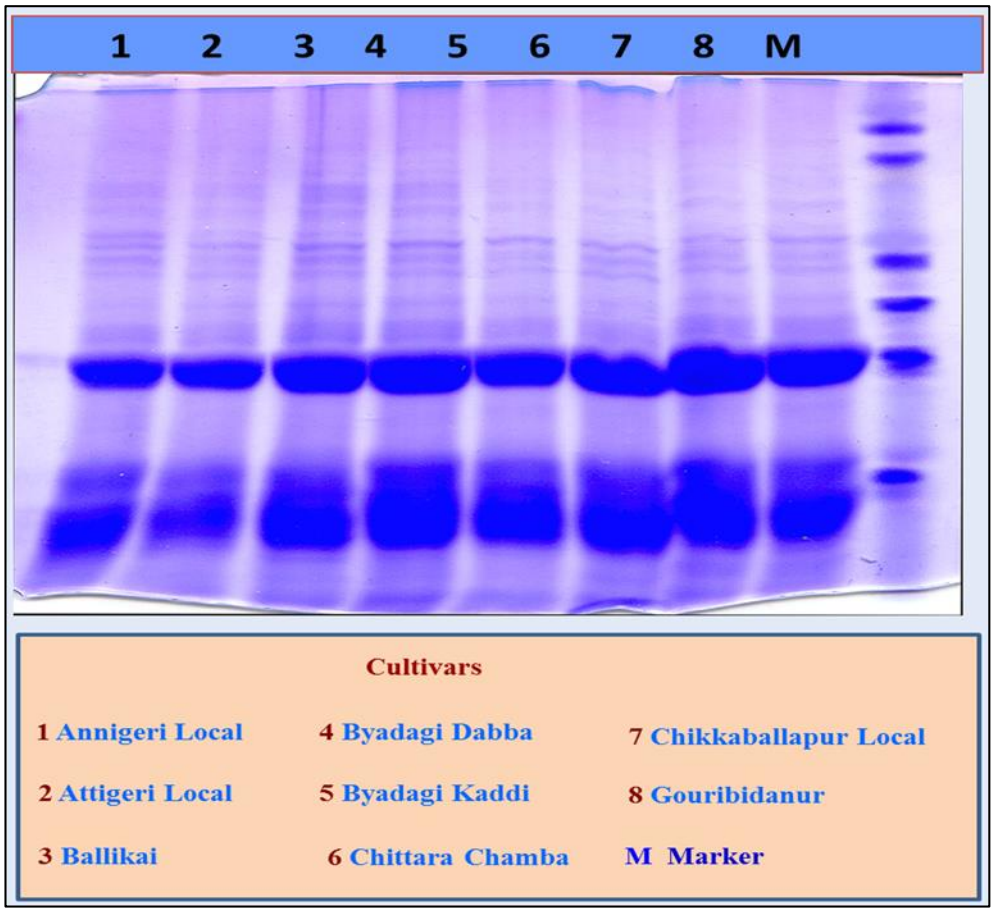

Fig 4: Electrophoretic banding pattern of different chilli cultivars

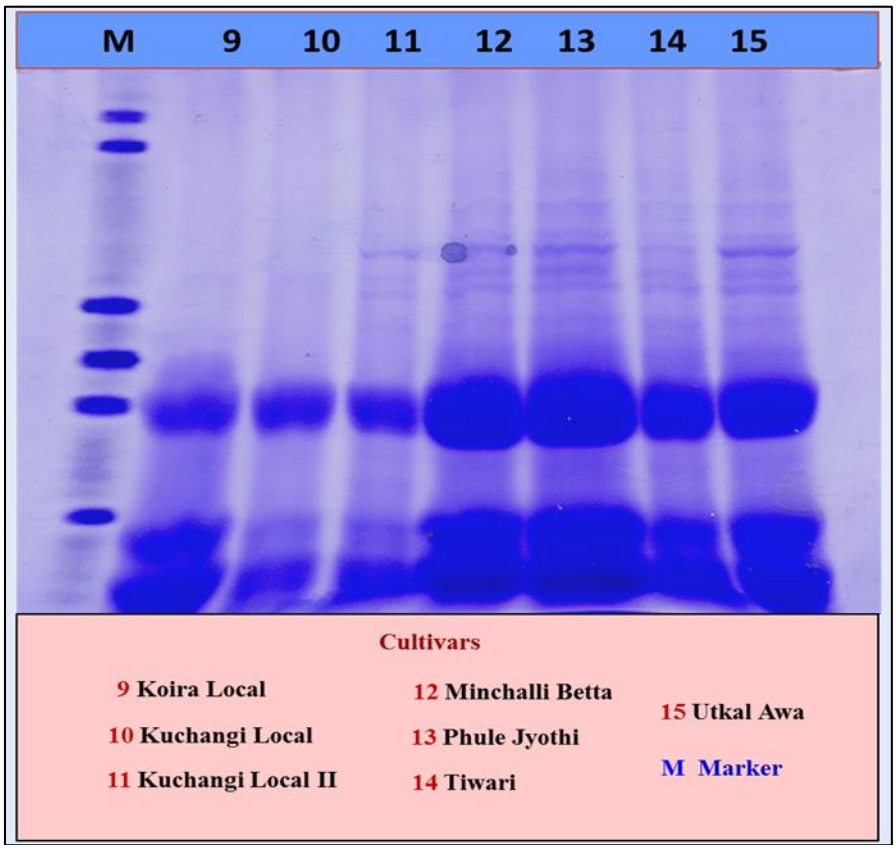

Fig 4: Electrophoretic banding pattern of different chilli cultivars

Conclusion

Keys developed using Standard Phenol and Modified phenol test with 0.5 per cent $CUS0_4$ and 1 per cent $FeSO_4$ test showed distinct characteristic reaction patterns and identified nine cultivars distinctly out of fifteen evaluated, however failed to identify six cultivars. However, SDS-PAGE of total soluble proteins clearly distinguished all the cultivars by unique protein profile and aided in identifying them individually and distinctly. Qualitative expression of colour reaction by chemical test can group cultivars and are time consuming. Thus the present studies envisage SDS-PAGE of total soluble proteins as a potential for rapid and reliable tool for characterizing, distinguishing and identifying chill pepper cultivars.

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