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Effect of seed treatment on germination and flavonoids diversity in accessions of butterfly pea (*Clitoria ternatea*)

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ABSTRACT

Butterfly pea (*Clitoria ternatea*) or aparajita is a medicinal herb with diverse medicinal properties including memory enhancing, antistress, anxiolytic, antidepressant, anticonvulsant, tranquilizing, and sedative properties. Various phytochemicals such as triterpenoids, flavonol glycosides, anthocyanins and steroids are reported in this species. Plant types with white and purple flowers are reported to occur in different regions of India. The phytochemical diversity with reference to geographical locations are not available for this species. Understanding the phytochemical diversity and characterization of genotypes are essential for commercial cultivation, conservation and future breeding programme. In the present study, we evaluated the phytochemical diversity of 19 accessions from different geographical regions from India in terms of flavonoid accumulation. Flavonoid content significantly ($p < 0.05$) varied in the species with geographical locations, and were in range from 1.0 ± 0.06 to 3.5 ± 0.1 mg/g of quercetin and 4.1 ± 0.08 to 8.5 ± 0.17 mg/g of kaempferol in dry leaf of *C. ternatea*. Highest kaempferol content was found in ODB-W; quercetin was highest in AP-B accession, while MH-T accession had the lowest kaempferol and quercetin among the accessions. Seed germination aspect of *C. ternatea* was studied as the plant belongs to a fabaceae family having seed coat dormancy and still there is a gap in availability of information on its seed germination. Effect of acid treatment on seed germination indicated that *C. ternatea* seed coat dormancy could be efficiently removed by treating with concentrated sulfuric acid for 10-15 min at 30°C.

Key words: *Clitoria ternatea*, Flavonoid accumulation, Seed germination

Clitoria ternatea L. (Butterfly pea) is an important medicinal plant with several medicinal qualities. The species is a native of the Caribbean, Central America and Mexico, but it is now naturalized in all over the tropics including India (Parrotta 2001). The roots, seeds and leaves of *C. ternatea* have long been widely used as a brain tonic and is believed to promote memory and intelligence (Mukherjee *et al*. 2008), body aches, especially infections, urinogenital disorders and as an anthelmintic and antidote to animal stings (Nirmal *et al*. 2008), indigestion, constipation, fever, arthritis and eye ailments, ascetics, enlargement of the abdominal viscera, sore throat and skin diseases (Anon. 1995), for the treatment of snakebite and scorpion sting in India (Kirtikar and Basu 1935). Different researchers have identified the several secondary metabolites, viz. flavonoids, terpenoids, ternatins and sterols etc. from *C. ternatea* (Mukherjee *et al*. 2008).

However, information on genetic and phytochemical diversity in *Clitoria ternatea* accessions is scanty (Bishoyi *et al.* 2014, Ali *et al*. 2013). Ali *et al*. (2013), reported the chemical variability among *C. ternatea* ecotypes collected from a relatively smaller geographical region group of five different places of India and analyzed based on only a single compound and reported considerable variability. However, no systematic effort has so far been reported to explore the major diversity areas of India with multi components of this species. Hence, the objective of this study was to characterize the chemical diversity from eleven native populations of *Clitoria ternatea* across Indian subcontinent to understand its chemical diversity.

Seed germination is a key stage of plant lifecycle and is influenced by internal factors like nature of seed coat, embryo or by the presence of inhibitors and also by environmental factors such as temperature, light, time after dissemination, soil moisture content etc. (Qu *et al*. 2008, Agarwal and Dadlani 1995). Blockage of entry of water due to the presence of waxy layer in the seed coat is a common cause of delay in seed germination or dormancy (Ballard 1973, Harrington 1916). Irregular germination and slow growth of the plants results in poor plant stand and increase the cost of production.

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Table 1 Effect of H_2SO_4 treatment on removing seed coat dormancy in *Clitoria ternatea*

Treatment	Treatment duration (min)	Incubation at 25ºC Mean±SD	Incubation at 30º C Mean±SD
T1-Control		57.78±7.7	57.78±20.4
T2	5	68.89±7.7	75.56±10.2
T3	10	95.56±3.8	100.00±0.0
T4	15	93.33±6.7	93.33±6.7
T5	30	100.00±0.0	100.00±0.0
T6	45	100.00±0.0	100.00±0.0
T7	60	100.00±0.0	100.00±0.0
T8	90	100.00±0.0	97.78±3.8

To overcome the problem associated with germination, seeds need to be subjected to a specific treatment for breaking dormancy, increasing of per cent and acceleration of uniform seed germination (Frett 1987). The various methods were described to remove seed coat dormancy; physical treatments like scarification, micro-fissuration or coat softening and embryo stimulation (pre-chilling and soaking) and chemical treatments to remove waterproofing or reducing content of inhibitors in the embryo or stimulate the growth etc. (Baskin and Baskin 1998, Bewley and Black 1994, Bradbear 1998, Herranzen *et al*. 1998, Miyoshi and Mil 1998). Impermeability of the seed coat to water is widespread in the legume family. Presence of hard seed coat makes germination either incompletion slow and non-uniform (Tigabu and Oden 2001). Pre-treatment with sulfuric acid was most widely used to soften seed coat and to enhance germination (Liu *et al*. 1981). In this study, we have standardized the seed treatment to enhance germination and assessed the flavonoids diversity of butterfly pea.

MATERIALS AND METHODS

The present investigation was conducted at ICAR-Directorate of Medicinal and Aromatic Plants Research (DMAPR), Boriavi, Anand, Gujarat, India lies in latitude of 22.5º and longitude 73.0º and having average annual rainfall of 800 mm, maximum temperature and minimum temperature ranges between 12.7º and 42º C. The experiment was conducted during the year of 2011-12. Cleaned and healthy seeds were selected for the germination study. Seed samples were collected from 11 different locations of India (Table 2), There were 19 different accessions of *C. ternatea* which was classified into two groups based on petal colour of the flower (Blue and white) which were presumed to have different phytochemical constituents. Seeds were collected from only one petal colour plant type from Maharashtra (POP 4), Bihar (POP 7), Asom (POP 8) and Tamil Nadu (POP 10). A characteristic pink (Lilac) shaded petal type, POP 6 (Odisha), and intermediate flower colour type, i.e. white petals with blue tinge on the petals (T), POP 4 (Maharashtra) and POP 8 (Asom) were also included in the study. The seeds were germinated in polybags and seedlings were transplanted in main field. Leaf samples were harvested randomly from three plants of each accessions at 9 months after planting. Samples were cleaned and dried at 60°C temperature and made into fine powder using 2cyclone mill. The standard chemicals were kaempferol and quercetin. Ultra-pure deionized water was used for reagent preparations.

Table 2 Flavonoid accumulation among the different geographical samples of *Clitoria ternatea*

Locality	Population code	Accessions	Flower colour	Quercetin content mg/g (DW)	Kaempferol content mg/g (DW)
Udaipur,Rajasthan, Northern India	POP 1	RAJ-W	White	3.005 ± 0.14^{c,d}	7.924 ± 0.22^{c,d}
		RAJ-B	Blue	3.044 ± 0.20^{c}	8.154 ±0.35^{b,c}
Anand,Gujarat, Western India	POP 2	GUJ-W	White	1.360 ± 0.12^{i,j}	6.066 ± 0.17^{g,h}
		GUJ-B	Blue	1.690 ± 0.10^{h}	5.894 ± 0.08^{h}
Mandsaur,Madhya Pradesh, Central India	POP 3	MP-W	White	3.299 ± 0.17^{a,b}	8.074 ± 0.17^{b,c,d}
		MP-B	Blue	2.991 ± 0.14^{c,d}	7.935 ± 0.28^{c,d}
Akola, Maharashtra, Western India	POP 4	MH-T	White with blue tinge	0.995 ± 0.06^{k}	4.087 ± 0.08^{j}
Kalyani,	POP 5	WB-W	White	2.822 ± 0.09^{d,e}	7.781 ± 0.09^{d}
West Bengal, Eastern India		WB-B	Blue	3.020 ± 0.14^{c,d}	7.957 ± 0.35^{b,c,d}
Bhubaneswar,Odisha, Eastern India	POP 6	ODB-W	White	3.127 ± 0.10^{b,c}	8.501 ± 0.17^{a}
		ODB-B	Blue	2.728 ± 0.04^{e}	7.023 ± 0.17^{e}
		ODG-L	Lilac	2.139 ± 0.12^{g}	6.216 ± 0.21^{g,h}
Pusa, Bihar, Eastern India	POP 7	BH-B	Blue	3.128 ± 0.12^{b,c}	7.801 ± 0.10^{d}
Gauvahti, Asom, Eastern India	POP 8	AS-T	White with blue tinge	1.242 ± 0.19^{j}	4.375 ± 0.35^{j}
Venkataramannagudem,Andhra Pradesh,	POP 9	AP-W	White	2.458 ± 0.06^{f}	6.279 ± 0.10^{g}
Southern India		AP-B	Blue	3.489 ± 0.12^{a}	8.268 ± 0.12^{a,b}
Coimbatore, Tamil Nadu, Southern India	POP 10	TN-W	White	1.501 ± 0.22^{h,i}	5.239 ± 0.02^{i}
Ernakulum, Kerala, Southern India	POP 11	KL-W	White	1.965 ± 0.14^{g}	6.241 ± 0.09^{g}
		KL-B	Blue	2.644 ± 0.04^{e,f}	6.637 ± 0.13^{f}

Values are expressed as Means ± Standard deviation; (n=3). Different superscripts alphabets showed the significant differences ($P < 0.05$) in the same column, and results are represented in descending order; a > b >c > d > e > f > g > h >i> j > k.

Sulfuric acid used was of analytical grade. Chromatographic grade methanol, acetonitrile and formic acid were used for analysis.

Seeds were kept in concentrated sulfuric acid for 5, 10, 15, 30, 45, 60 and 90 minutes and washed thoroughly in running tap water to remove the acid traces and subsequently seeds were kept overnight soaked before germination test. Each replication had 15 seeds and arranged on moistened filter paper lined petri plates and kept in an incubator at two different temperature of 25º and 30º C. Counts on germination was recorded from each treatment at one day interval up to 3 days. Control seeds did not receive acid treatment. The germination percentage was calculated using the formula as; (% germination= total number of seed germinated out of 15 seed after 3 days/ 15 × 100) and the germination percentage increased over control was determines as the formula mentioned below. Each treatment was replicated thrice.

$$\text{Germination percentage increased over control} = \frac{(\text{Seed germinated after treatment}) - (\text{Seed germinated in control})}{\text{Seed germinated in control}} \times 100$$

The standard stock solution (1 000 ppm) was prepared by weighing exact 5 mg of each standard (kaempferol; 97.0% and quercetin; 95.0%) in 5 ml methanol. The kaempferol and quercetin content was analyzed in the plant extracts followed by acid hydrolysis (Xu *et al*. 2012). The leaf extract were prepared by weighing 10 g of dried homogenized sample powder into a 250 ml round bottom flask and added with 70 ml of 80% methanol. The resulting mixture was heated for 60 min on a water bath at 80 ± 1 p C temperature. Cooled extract was vacuumed filtered through a filter paper and centrifuged at 1 0000 rpm for 10 min to remove any floating matters. For hydrolysis, sample extract (12 ml) was treated with 5M HCl (8 ml) at 80º C on a water bath for 30 min. The sample was cooled and diluted with methanol and filtered through 0.45 µm membrane filter prior to chemical analysis. All measurements were carried out in triplicate. Compound yield was represented as milligrams per gram of dried extract.

Analyses were performed on a reverse phase C18 Altima column (4.6 × 250 mm, 5 µm) using a high performance liquid chromatography; HPLC SPD-20AD model equipped with a binary solvent delivery pump, an auto sampler coupled with UV-visible detector. LC solution software was used for data acquisition. Column temperature was maintained at 40º C and the wavelength was set at 370 nm. Separation was achieved in the mobile phase used as an isocratic elution of acetonitrile (45%) and 0.1% formic acid in water (55%). The injection volume was 10 µL.

All the experimental data were analyzed in triplet and the values were presented in mean and standard deviation (± SD). In these seed germination studies, the experiment was planned by completely randomized design and treated in three replications of each treatments (N=9). The data collected were statistically analyzed by the procedure described by Panse and Sukhatme (1967). In the experiment of flavonoid accumulation among 19 accessions of *C. ternatea;* the resulting data were analyzed by using WASP (Web Agri Stat Package, version 2) software (http://icargoa.res.in/wasp2.0/index.php). A significant difference was calculated at 0.05 level.

RESULTS AND DISCUSSION

Effect of seed treatment on germination

Seed dormancy is nature's way of setting a time clock that allows seeds to initiate germination when conditions are normally favorable for germination and survival of the seedlings. *C. ternatea* is having seed coat dormancy and its existence has been reported for more than 100 years, which infused the seed germination under natural conditions studied by various group of researchers (Ainouz *et al*. 1994, Cabrales and Bernal 1981, Guppy 1912). Hence, this investigation for the effective seed germination in *C. ternatea*, eight different duration of acid treatment were studied and the results revealed that it significantly enhanced seed germination over control (Fig 1). Mechanical scarification treatment also effective in seed germination as reported by Mackay *et al*. (2001) since irregular germination was observed due to seed scarification and it was not included dormancy breaking in the present study. Like many other physiological processes, seed germination is temperature dependent and the optimal level of temperature for germination varies considerably between species (Demel 1996). To achieve optimal generation, it is necessary to have knowledge of the sub and supra optimal temperature limits. Two different incubation temperature of 25º and 30º C was tried to enhance the germination and temperature did not appreciably influence treatment effects since both the incubation temperatures were equally efficient in enhancing the seed germination in the species. However, incubation at 30ºC caused faster seedling emergence and growth (Fig 1) compared to the incubation temperature of seeds at 25º C.

Seed germination data recorded in daily basis showed the germination started after 24 h in all investigated treatments and the maximum germination rate was found at 2 DAT (days after treatment) in seeds kept at both temperature 25º and 30º C. The process was completed within three days after treatment except in control in which the germination continued even after 5 DAT in both the incubating temperatures. Percentage of germination in acid treated seeds increased over control varied from 19.20 to 73.10 for 25º C incubation and similarly it varied from 30.8 to 73.1 for 30º C incubated seeds (Fig 1). Seed germination was 100% in acid treatments for 10, 30, 45, 60 and 90 min, however it was a par with acid treatment of seeds for 15 min. Acid treatment of seeds for 5 min also improved seed germination; however it was not at par with other superior treatments. The study also showed that prolonged treatment of seeds in acid even though improved seed germination, but affected the quality of seedling emergence. Treatment of seeds in acid for 15 min onwards caused the seedling damage

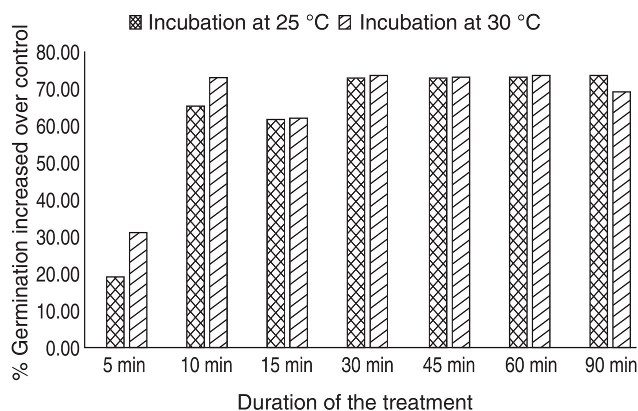

Fig 1 Germination percentage increase over control after seed treatment in acid under different durations

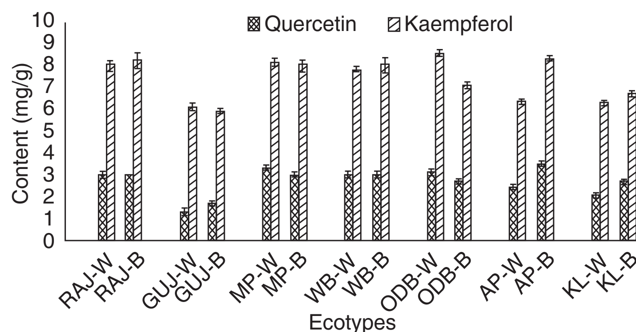

Fig. 2 Flavonoid accumulation among the two different floral variants; white flowered and blue flowered *Clitoria ternatea* collected from different geographical regions

and yellowing of the cotyledons (Fig 1). Hence, it was inferred that in *C. ternatea*, effective seed germination can be achieved by treating seeds with concentrated sulfuric acid for 10-15 min (Table 1 and Fig 1).

Flavonoid accumulation in Clitoria ternatea *among accessions*

Hydrolyzed leaf extractof *C. ternatea* was applied for HPLC analysis to investigate compounds, viz. quercetin and kaempferol content and the compounds were separated in retention time of 4.26 and 5.16 min, respectively. Identity of these compounds in plant extract was positively confirmed by comparing with standards and spiked samples. A qualitative information by the chromatogram comparison of all analyzed samples revealed a similar chemical fingerprint which suggesting the presence of quercetin and kaempferol in the explored ecotypes. Moreover, the peak intensity showed that higher quantity of kaempferol as compare to quercetin in all sample analyzed.

In a quantitative approach, the aim of this study was to assess the phytochemical variations in the different accessions collected from different regions of India. Significant ($P < 0.05$) difference of flavonoids (quercetin and kaempferol) was observed between the 19 ecotypes of *C. ternatea* from different origins (Table 2). In a similar study, Deng *et al*. (2015) reported variations in flavonoid content among 12 genotypes of *Cyclocarya paliurus*. Phytochemical variability in genotypes occurring in various geographical locations are the results of genotypic and environmental interactions (Sanwal *et al.* 2013, Deng *et al.* 2015). Table 2, shows the distribution of kaempferol in accessions which was ranging from 4.087 ± 0.067 to 8.501 ± 0.069 mg/g, the finding was accompanied with a wide ranged kaempferol variability in *Clitoria ternatea* genotypes collected from five different Indian locality (Ali *et al*. 2013). ODB-W accession had the highest yield of kaempferol (8.501 ± 0.069 mg/g) which was followed by 8.268 ± 0.060 mg/g (AP-B). However, the samples corresponding to MH-T accession had the lowest values as 4.087 ± 0.067 mg/g for kaempferol. Quercetin content ranged from 0.995 ± 0.018 to 3.489 ± 0.016 mg/g in the studied ecotypes. This result was accordance with the study conducted by Kosakowska *et al*. (2015) in *Tilia cordata.* The study reported variability in quercetin content among the sample collected from 19 sites from Poland. The highest quercetin amount was detected in AP-B (3.489 ± 0.016 mg/g) and MP-W (3.299 ± 0.028 mg/g). MH-T accession had the lowest kaempferol content, also had the poor yield of quercetin (0.995 ± 0.018 mg/g). In this experiment, both these compounds were found in lower amount from MH-T and AS-T accessions; while maximum content was found in ODB-W, AP-B and MP-W accessions.

Plant morphotypes always presumed to have different phytochemical constituents and hence preference given to specific morphotypes over others for medicinal uses. More scientific proof in terms of phytochemical variability will be useful to make informed choices in choosing specific plant types for specific medicinal use. Petal colour variability in *C. ternatea* was reported by Bishoyi and Geetha (2012). Among them, white flowered variety is believed to be therapeutically more potent and blue petal morphotype is considered to less potent (Mukherjee *et al.* 2008). Information on phytochemical diversity in *C. ternatea* morphotypes is lacking. Knowledge on the chemical diversity is crucial for its therapeutic usage. Present work mainly aimed to identify the diversity of flavonoid compounds among different floral morphotypes collected from diverse locality of India. The phytochemical data is presented in Fig 5. Interestingly, flavonoid yield from blue and white petal plant types within same population varied widely. It was noticed that there is no relationship between the petal colour and flavonoid content among the accessions studied. The results showed that no petal colour plant type is superior to others in terms of the phytochemicals (kaempferol and quercetin) distribution. These results are consistent with observations of Ali *et al*. (2013) on kaempferol variability in five populations having different floral types of *C. ternatea.*

The majority of medicinal plants are collected from wild and information on phytochemical variations in different geographical origins are not available for most of them. In present study, we report the variations in two bioactive flavonoids i.e. quercetin and kaempferol in *C. ternatea* leaf samples collected from 19 different geographical regions of

India. The results revealed that flavonoid accumulation significantly differed among the accessions studied. The content of kaempferol was in higher order compared to quercetin in this species. Both white and blue petal plant types possessed similar phytochemical profile and variations in distribution was attributed to geographical locations rather than the petal colour. Seed germination studies showed that effective seed germination was achieved by sulfuric acid treatment for 10 min, however longer duration of acid treatment caused the seedling damage and yellowing of the cotyledons.

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