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Aspartic protease gene expression and kaempferol production at different germinating stages of cow pea (*Vigna articulata* L.) seeds varieties

Pratibha Chaurvedi*, Shradha Surve, Sandeepan Mukherjee, Shreewardhan Rajopadhye, Nikhil Kasarpalkar, A. Rosalind Marita and Abhay Chowdhary

Haffkine Institute for Training, Research and Testing, Parel, Mumbai

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ABSTRACT

Legumes including cowpea have been widely grown and their seeds are used as human and animal food to provide calories and protein. As food, cowpea seeds are eaten in different forms; they could be boiled, parched, fried, roasted, mixed with sauce or stewed and consumed directly. Its seeds are consumed in different forms as they provide important vitamins, phyto-nutrients including antioxidants besides carbohydrates, minerals and trace elements. In addition, it is a cheap source of high quality protein in the diets of millions in developing , who cannot afford costly animal protein for balanced nutrition .The present study describes the probable role of Aspartic Protease gene expression in kaempferol production in germinating cow pea seeds .The various germinating stages of two different varieties of Vigna articulata L. namely Pusa Kolum and Konkan Sadabahar seeds were used as the experimental material. The drought resistance nature of Aspartic protease stimulate the production of Kaempferol , which is a well-known antioxidant compound . The production of antioxidant compound i.e. Kaempferol was decreased with the germination time which has been also seen in gene expression of Aspartic protease in Konkan Sadabahar ,which support the principle of production of secondary metabolite at the time of stress .The Kaempferol content were analysed with the help of HPTLC spectral studies whereas gene expression were done by molecular methods. This kind of study is not well documented in Vigna articulata.

Key words: *Vigna articulata*, Pusa Kolum, Konkan Sadabahar, Kaempferol, Aspartic Protease, Gene Expression

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INTRODUCTION

Plants have several popular regulatory metabolitesto control the flower color, flavoring factors, pollination, pollen tube germination, seed maturationand seed coat browning, seeds and spore germination, plant growth and development andestablish themselves against biotic and abiotic stressconditions. Flavonoids are a large group of low molecular weight, ubiquitously distributed, polyphenolic secondary metabolites. Thesecompounds play a significant role in various stages of plant growth and their existence in theenvironmental stresses.Flavonoids are a large subgroup of secondarymetabolites categorized as phenolic compounds, widely disbursed throughout plants and prokaryotes [1, 2]. More than 6,500 flavonoids have been identified [3]. They protect plants againstvarious biotic and abiotic stresses and exhibit adverse spectrum of biological functions and play animportant role in the interaction between the plantand their environment[4] it absorbed theharmful UV radiation induced cellular damage [5].Flavonoids are not essential for plant survival, but they are bioactive and influence thetransport of the plant

hormone, auxin [6]. Apart from that,they are responsible for flower colors, protecting the plantsfrom microbes and insects [7, 8, 9].

Plants use complex recognition and response mechanisms to protect themselves from pathogen attack. Major resistance (*R*) genes specify recognition of pathogens carrying the corresponding avirulence genes leading to the rapid activation of a battery of inducible defenses, including reinforcements of the cell wall, synthesis of phytoalexin antibiotics, and deployment of pathogenesis-related proteins such as chitinases and glucanases [10,11]. Keampferol is a strong antioxidant and helps to prevent oxidative damage of our cells, lipids and DNA. Kaempferol seems to prevent arteriosclerosis by inhibiting the oxidation of low density lipoprotein and the formation of platelets in the blood. Studies have also confirmed that kaempferol acts as a chemopreventive agent, which means that it inhibits the formation of cancer cells.[6,8]

The diverse roles of plant proteases in defence responses that are triggered by pathogens or pests are becoming clearer.Some proteases, such as papain in latex, execute the attack on the invading organism. Other proteases seem to be part of asignalling cascade, as indicated by Protease inhibitor studies. Many plant Aspartic Proteases contain an additional sequence of ~100 amino acids termed the plant-specific insert, which is involved in host defense and vacuolar targeting(12).Of practical interest among plant APs have their roles in plant pathogen resistance (13) as well as in senescence and postharvest physiology [14,15**].**

Vignaunguiculata (Cowpea) is drought tolerant grain legume which has great agronomic interest as food and fodder.The grain constitutes an important source of dietary protein and secondary staple carbohydrate Apart from this the seed are rich in flavonoid such as kaempferol. As it is a dought tolerant grain and all the properties are correlated to the resistance mechanism of the plant ,hence in the present investigation to find out the correlation between the these all ,the kaempferol content and the gene expression of aspartic protease were analysed in different germinating stages of two varieties of cow pea seeds. This study is the first kind of report ,that is not well documented in literature also describes the nutritional value of germinating seeds of *Vignaunguiculata* (Cowpea).In the present investigation ,we have selected two varieties of cowpea namely Konkan Sadabahar and PoosaKolum, which are the more popular varieties in Maharashtra.

MATERIALS AND METHODS

Two varities of *Vignaunguiculata*, seeds namely Pusa Kolum and Konkan Sadabahar were collected from the Agricultural University of Dapoli, Maharashtra and kept them for germination from one day to seven days separately. The fresh germinating tissues of all the samples were subjected to examine the relative gene expression of enzyme “Aspartic Protease”separately.The total RNA extraction were carried out using the protocol of RNASure Plant Kit (Genetix)and Conversion of mRNA into cDNA was done by using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fast PCR master mix (SapphireAmp)The amplification of gene Aspartic protease ware done by using primers Forward primer : 5’-AGATGCACCTTTTCGCTTGC-3’ and Reverse primer : 5’-GAAGAGATATCCCCGCAGCC-3’.The PCR Condition were standardized as follows

Table 1. PCR condition

Step	Temperature (°C)	Time
Initial denaturation	95	5 mins
Denaturation	94	1 min
Annealing	61	30 sec
Elongation	72	30 sec
Final extension	72	7 min
Hold	4	As applicable

Agarose Gel Electrophoresis

100 ML 5X TBE buffer was prepared which contained 5.4g Tris base, 2.7g Boric acid 0.5M EDTA. EDTA was prepared separately by mixing 18.612g EDTA in 100ml distilled water. A 2% agarose gel was used for electrophoresis of PCR products. EtBr was used as a staining agent and was mixed with the agarose before casting the gel while loading, 5µl sample was loaded on the gel without mixing with dye as the PCR Master Mix contains the dye already mixed in it.The electrophoretic unit was allowed to run at a constant voltage of 80V till the dye front

reached three fourth of the gel caster.The power pack was then switched off and the gel was observed under Gel-Doc.

Kaempferol extraction and it'sHigh Performance Thin Layer Chromatography (HPTLC) analysis

The two varieties of Cow pea were germinated separately *in vivo* condition and their fresh tissue were subjected for Kampferol extraction by using the methanol cold extraction method .The extracts were dried *in vacuo* and subjected for it's kaempferol content analysis by using HPTLC spectral studies separately with standard reference compound of kaempferol in Anchrome Pvt. Ltd. Mulund, Mumbai. Toluene : Ethyl acetate : Methanol : Formic acid (30:15:1:2)was used as mobile phase in Instrument CAMAG Linomat 5 "Linomat5_080222" S/N 080222 (1.00.13) on 20.0 x 10.0 cm HPTLC plates silica gel 60 F 254. We have consider only day one, day four and day seven old samples for all the studies**.** The Rf value of kaempferol in the mentioned solvent system was 0.39 ,which was derivatized by 5% ethanolic HCl, which gave the characteristic yellow color.

RESULTS AND DISCUSSION

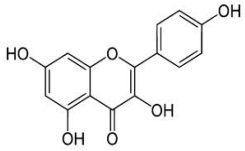


Fig.1. Structure of Kaempferol

Legumes including cowpea have been widely grown and their seeds are used as human and animal food to provide calories and protein. As food, cowpea seeds are eaten in different forms; they could be boiled, parched, fried, roasted, mixed with sauce or stewed and consumed directly. Its seeds are consumed in different forms as they provide important vitamins, phyto-nutrients including antioxidants besides carbohydrates, minerals and trace elements. In addition, it is a cheap source of high quality protein in the diets of millions in developing , who cannot afford costly animal protein for balanced nutrition [16]Two varities of *Vignaunguiculata* namely Pusa Kolum and Konkan Sadabahar were germinated *in vivo* condition.(Fig.2.)

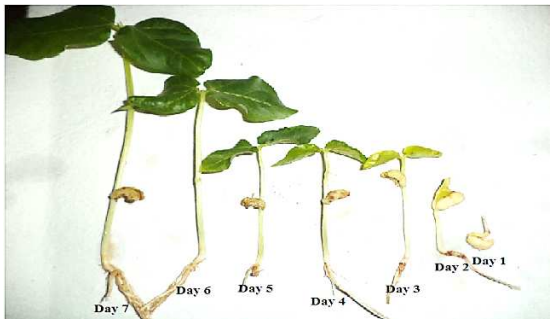


Fig 2.: Different stages of *Vignaunguiculata* seeds germination

Table 2.:Kaempferol content% in Konkan Sadabahar(KS) and Poosa Kolam(PK) seeds of *Vignaunguiculata* at different stages of germination. ± mean SD of triplicate Significant p value ≤ 0.001(Anova test)

Sr No.	Name of Explant	Kaempferol content (%)
1.	KS control	1.99±0.031
2.	KS day 1	0.87±0.053
3.	KS day 4	0.311±0.045
4.	KS day 7	0.309±0.062
5.	PK control	2.42±0.63
6.	PK day 1	0.716±0.067
7.	PK day 4	1.205±0.073
8.	PK day 7	0.452±0.053

The Kaempferol estimation of various samples of Konkan Sadabahar(KS) and Pusa Kolum(PK)varieties i.e. KS Control,KS day 1,KS day 4,KS day 7 ,PK control, PK day1,PK day 4,PK day 7 were carried out separately . The analysis was done with the help of High Performance Thin Layer Chromatography (HPTLC)analysis with that of the standard reference compound of Kaempferol. Anova Test was used for statistical analysis(Table 2.) .

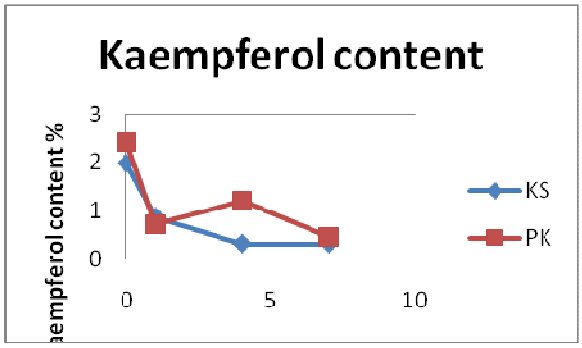
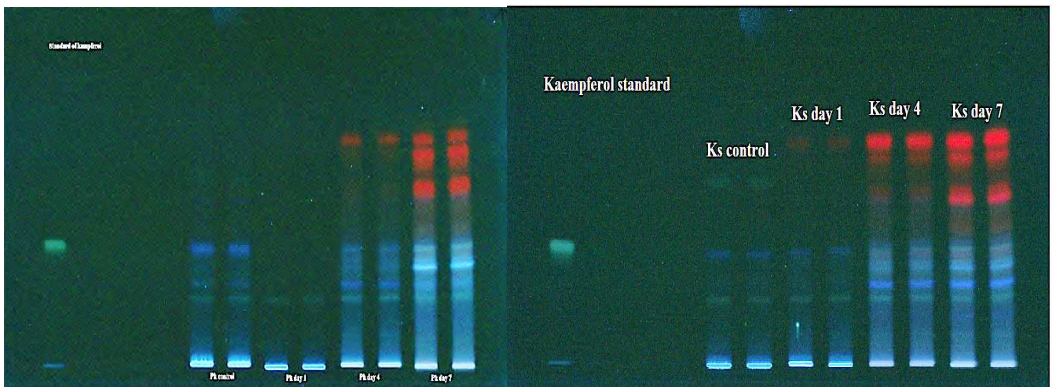
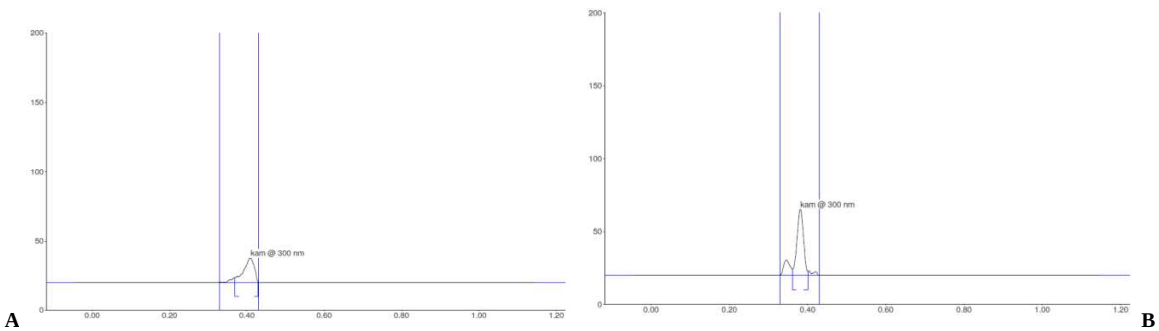


Fig.3 Kaempferol content (%) at different germinating stages of two varieties KonkanSadabahar(KS) and PusaKolum(PK) of *Vigna articulata L.*



A **B**

Fig 4.: HPTLC fingerprinting of methanolic extract of *Vignaunguiculata* at 366 nm. (A-Pusa Kolum; B-Konkan Sadabahar) for kaempferol content with the reference compound of kaempferol at different stages of germination



A **B**

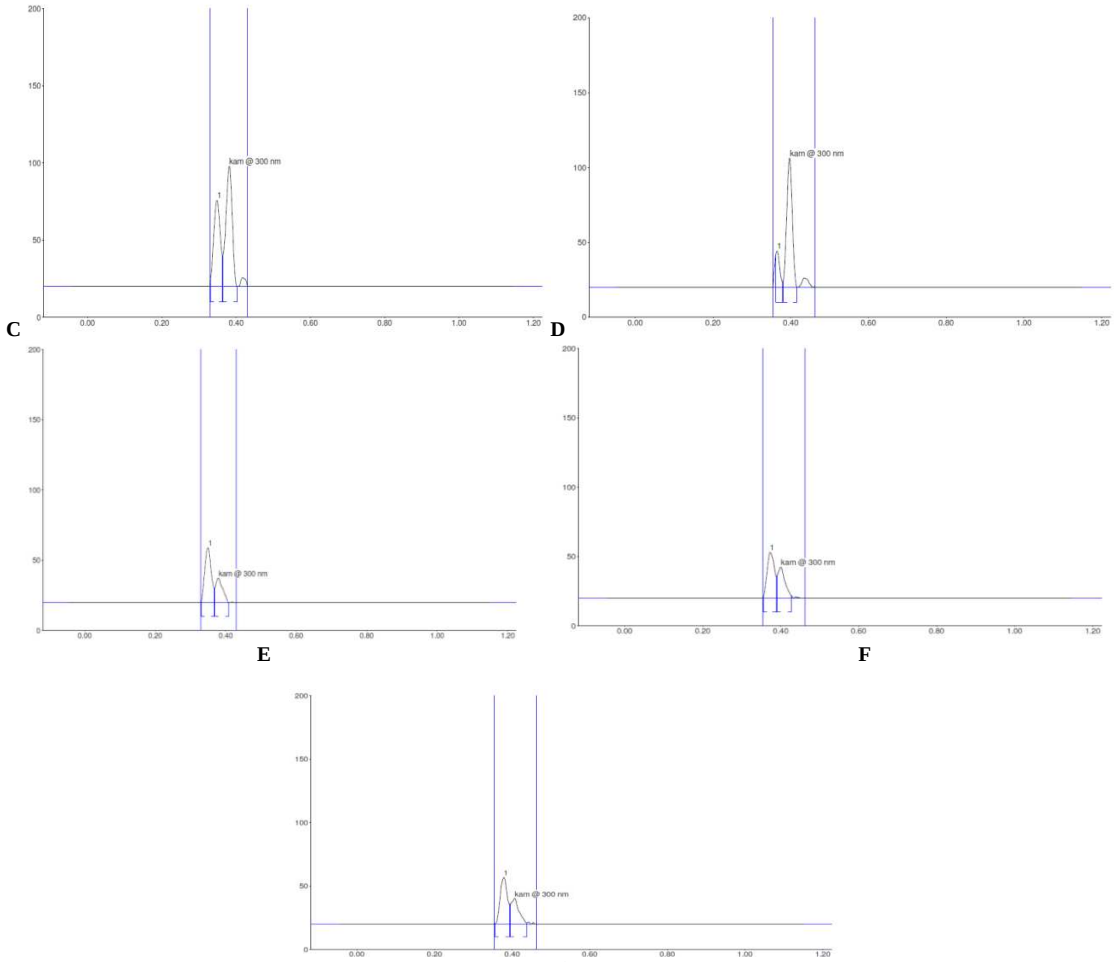


Fig5.:High Performance Thin Layer Chromatographic (HPTLC) chromatogram showing kaempferol content (A-Standard) present in Poosa Kolum(B-D), Konkan Sadabahar (E-G), at different stages of seed germination

Two varieties of cowpea (*Vignaunguiculata*) have been germinated up to 7 days and kaempferol content were examined by HPTLC analysis(Fig 5.) at the age of 1st,4th and 7thday of germination with that of the standard reference compound of kaempferol separately. The kaempferol content were calculated with the help of peak areain respective chromatogram with standard compound of kaempferol. HPTLC chromatographic analysis results depicts that as the seeds germinates the amount of kaempferol changes. The maximum content of kaempferol in germinating explants was in PK at day 4 of germinating stage(1.205%) among allexplants used . In both the varieties the kaempferol content was maximum at fourth day of germination .(Fig 2 ;germinating stages of variety Pusa Kolum) As showing in the figure ,the sudden change in the content of chlorophyll directly effect the photosynthetic capacity of the plant ,which in turn effects the production of the glucose. The Sugars act as signaling molecules, whose signal transduction pathways may lead to the activation or inactivation of gene expression. Besides mRNA accumulation, sucrose affects production and accumulation of flavonoid. The enhancement of kaempferolin fourth day of germination of seeds might be due to this reason. In the literature it is reported that kaempferol works as growth hormone[17],which suggests the in the zero growth stage it is in highest content,but when the germination started it becomes consume ,hence the kaempferol concentration gradually decreases as the days of germination increases. It has been reported that enzyme Aspartic Protease acts as drought resistance agent (15)since it's expression is correlated to it's resistance property, the secondary metabolite production becomes stimulated due to production of phytoallexin, which in turn favors the the production of kaempferol.

Gene expression of enzyme aspartic protease was checked with the help of PCR analysis in two varieties of *Vignaunguiculata*, Namely Pusa kolum and Konkan sadabahar. The relative quantity of gene expression in Pusa

Kolum was maximum at six day of germination(1.135),whereas in Konkan Sadabahar it was maximum at the first day of germination(8.054774).This variation may be due to the different genetic makeup of the two varieties and involvement of some mutation orchromosomal aberration which has a impact on gene expression of Aspartic protease enzyme.(Fig. 6,7,8,9).

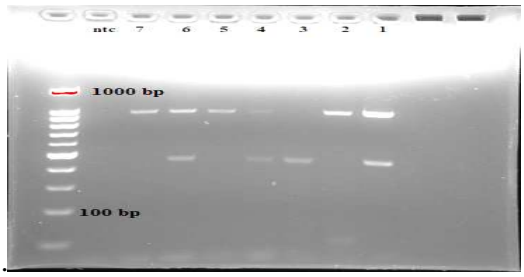


Fig 6 : Pusa Kolum gel pic : Well 1- 100bp ladder , Well 2-ntc, well 3- day 7, Well 4- day 6, Well 5- day 5, Well 6- day 4, Well 7- day 3, Well 7- day 2, Well 8- day 1

Table3.showing the band relative quantity of Aspartic protease gene expression in Pusakolum seeds varieties of *Vignaunguilata*

Lane Number	Relative Quantity
Lane 3	1
Lane 4	1.135
Lane 5	0.758
Lane 6	0.224
Lane 7	0
Lane 8	0.108
Lane 9	5.038

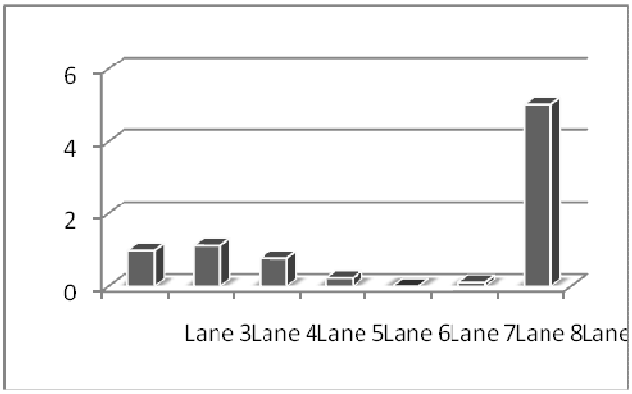


Fig 7. : graph showing gene expression during different stages of germination in pusakolum

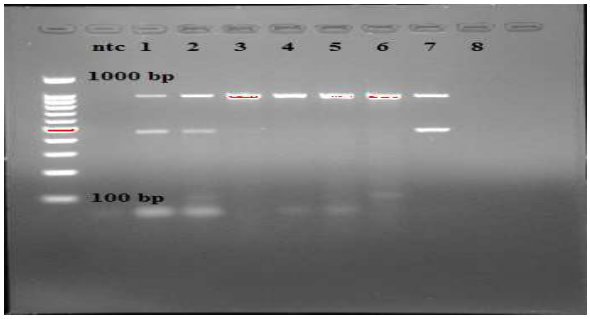


Fig 8 : Konkansadabahar gel pic : Well 1- 100bp ladder , Well 2-ntc, well 3- day 7, Well 4- day 6, Well 5- day 5, Well 6- day 4, Well 7- day 3, Well 7- day 2, Well 8- day 1

Table4 : Relative quantity of the band

Lane Number	Relative Quantity
Lane 3	1
Lane 4	2.006
Lane 5	8.531986
Lane 6	4.573648
Lane 7	7.425138
Lane 8	8.054774
Lane 9	2.972642

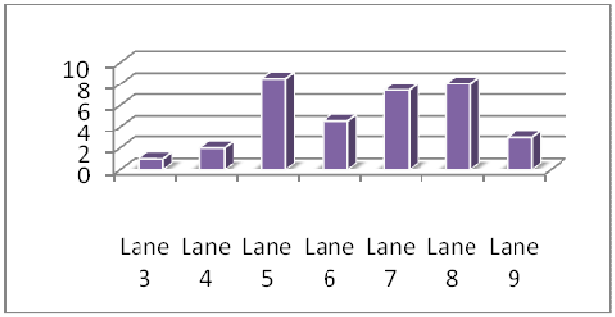


Fig 9: graph showing gene expression during different stages of germination in Konkan Sadabahar

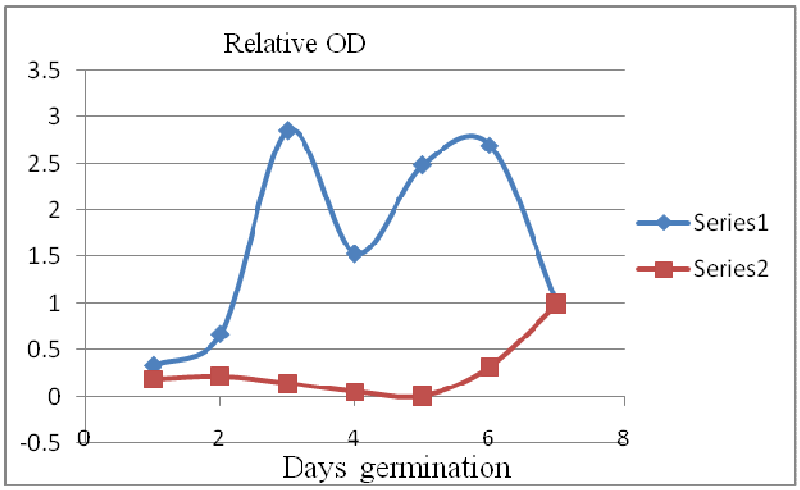


Figure10.Comparative gene expression of Aspartic protease in Pusa Kolum and Konkan Sadabahar at different germinating stages

CONCLUSION

Secondary metabolites are manufacturing in plants due to stress response and is already been well studied by many scientist[18-33].It was concluded that Kaempferol ,which is antioxidant flavonoidal compound has been checked at the time of different germination stages of two varieties of Cowpea seeds (Pusa Kolum and Konkan Sadabahar). A probable correlation between the genetic expression of Aspartic Protease enzyme and kaempferol production and accumulation in these samples was found ,which also suggested that due to the drought resistance nature of Aspartic protease ,it's expression stimulate the kaempferol production between fourth day and sixth day of germination ,which is the right time to consume that for it's beneficiary effect because till that time the Kaempferol content remain maximum ,after that a decline it's content was observed.This kind of study is not well documented in *Vigna articulata*.

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