CHAPTER-VI

INVESTIGATION OF PEPTIDES OF *CICER ARIETINUM* SEEDLINGS AND THEIR ROLE IN HORMONE ACTION

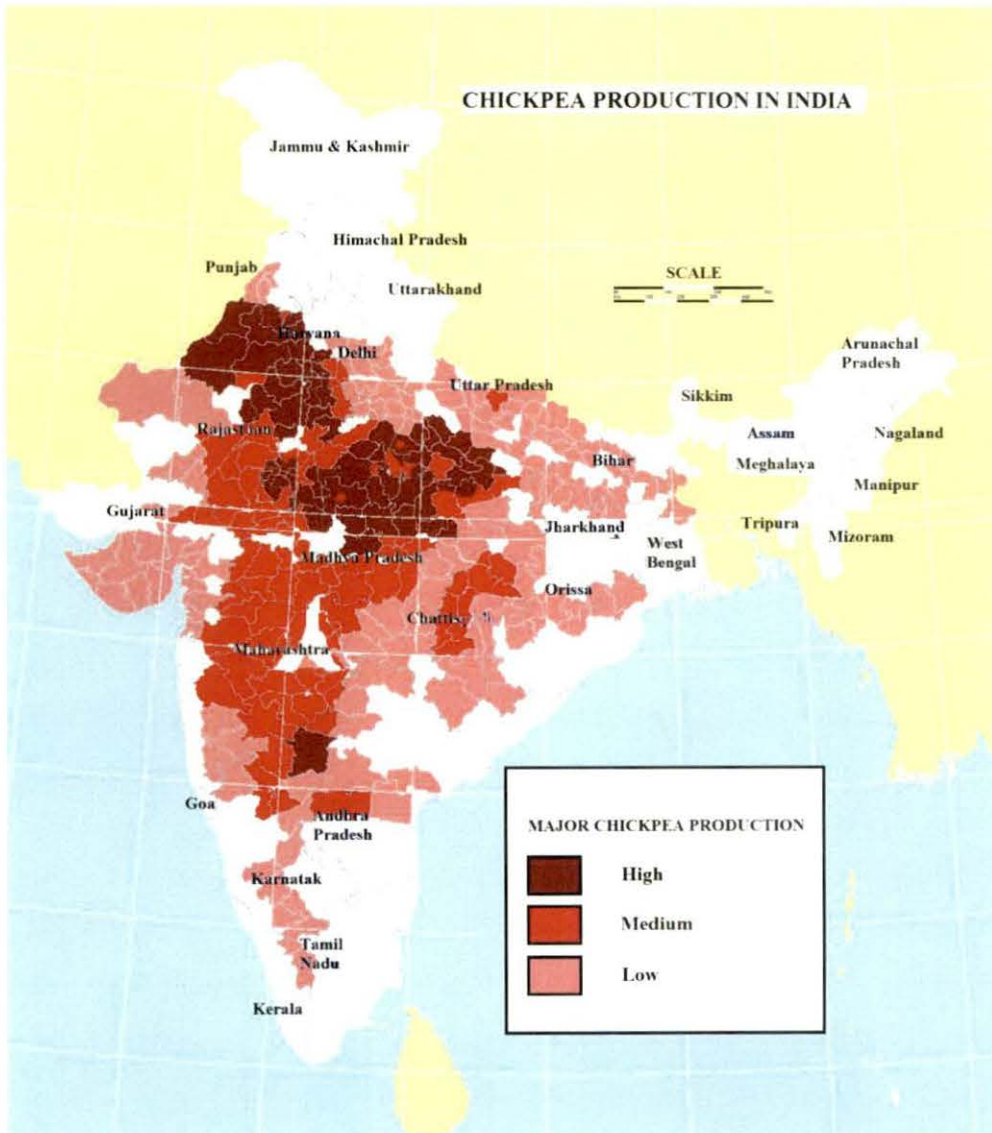

Figure 6.1 Annual production of chickpea in India

6.1 INTRODUCTION

Chickpeas (*Cicer arietinum* L.) were first introduced in the Middle East countries nearly about seven thousand million years ago (Ladizinsky and Adler, 1976). It is stress tolerant crop of the family Leguminosae (Fabaceae) and is used in various food preparations in several developing countries. Commercially two types of chickpea cultivars are produced worldwide: 'Desi' and 'Kabuli'. 'Desi' type seeds are smaller and dark coloured with thick seed coat. 'Kabuli' chickpea seeds, on the other hand, are large, cream coloured with thin seed coat (Upadhyaya *et al.*, 2006). In Australia and Indian sub-continent, 'Desi' chickpea are primarily cultivated, whereas Canada produces both the types of cultivars and the remaining countries like Mexico, Ethiopia, US and Turkey mostly emphasize on the production of 'Kabuli' type chick pea. Globally 8.9 million tonnes of chickpea are produced and among this, more than 90% are consumed by producer countries itself.

Among pulses, chickpea is the premier crop in Indian subcontinent. Internationally, India is the largest producer and consumer of chickpea (Gaur *et al.*, 2008). India alone accounted for 69% of global chickpea production. A partial downward trend in chickpea production was observed in India and Australia during last decade, whereas Canada was the only country exhibited a major uplift in chickpea cultivation. In India, chickpea growing areas are about 6.67 million hectares, which represents 30% of the national pulse acreage. Production of chickpea has enhanced from 3.6 to 5.6 million tonnes during the last fifty years with annual growth rate of 0.58% (Agbola *et al.*, 2000). Mainly six states in India viz., Rajasthan, Maharashtra, Andhra Pradesh, Madhya Pradesh, Uttar Pradesh and Karnataka contributes 91% of total production and 90% of cultivated area of the country (Figure 6.1). A major change in chickpea production area has observed in recent past due to expansion of irrigation facilities in northern India. In northern states, chickpea cultivation area has reduced from 3.2 to 1.1 m ha due to its replacement with wheat and mustard, while the same area has increased from 2.6 to 4.3 m ha in south central states during last few decades. This creates a major and dynamic change in the chickpea area from long duration, cool, highly productive environment to short duration, warm, rain fed and less productive condition (Ramakrishna *et al.*, 2005). In this scenario, knowledge on the impact of quality or characteristic traits of chickpea marketing is of critical importance for scientists, exporters and farmers (Kelley, 1999). For achieving targeted production of

chickpea in next decades, horizontal expansion through crop diversification and strategic research on productivity enhancement is essential. But a large gap has always observed between the yield realized in experimental station and the output from actual farming. Inappropriate cultivation practices, low yielding and non-responsive genotypes against fertilizers, unmanaged pest and disease problem, lack of improved soil, poor rate of germination, stress sensitive high yielding cultivars and lack of appropriate support are the fundamental constraints, which are accountable for this un-trapped yield potential (Shakya *et al*, 2008).

Chickpea is a reliable source of protein for common people. In addition, chickpea is rich in minerals like phosphorus, calcium, magnesium, iron and zinc, dietary fibres, beta-carotene and unsaturated fatty acids (Jukanti *et al*, 2012). Soil fertility is also remarkably improved by cultivation of chickpea through fixing atmospheric nitrogen (Saraf *et al*., 1998). People are now well aware about the utility of this crop and the growing demand for chickpea can be efficiently mitigated either by improving the traditional cultivars through breeding or enhancing high physiological adaptability. Because of high price of seeds, efficient germination rate is of prime importance for the cultivation of this crop. But proper germination requires induction of α-amylase within seeds in appropriate time. Germination induced α-amylase and protease degrades complex starch and proteins respectively; and by this way these enzymes also reduce the dietary bulk through improving the digestibility of starch and proteins (Negi *et al*., 2001). In all cases the function of α-amylase is considered with top priority, through which the soluble sugars are mobilized by the breakdown of starch in germinating legume seeds (Fincher, 1989). Several authors demonstrated the role of phytohormones regulating α-amylase induction as well as germination in chickpea. Some studies indicated that gibberellic acid and kinetin enhanced the percentage of germination and seedling growth in polyethylene glycol induced water stressed chickpea seeds (Kaur *et al*., 1998). Exogenous application of gibberellic acid also enhanced α-amylase in chickpea during critical condition of water potential (Gupta *et al*., 1993). However the observed data indicated that indole acetic acid (IAA) inhibited growth and germination in stressed chickpea seedlings. Enhanced amylase activity was recorded in cotyledons and shoot of pea in presence of GA_3 and kinetin, whereas IAA reduced root amylase activity (Monerri *et al*., 1986). When overall carbohydrate metabolism is considered during germination, both GA_3 and kinetin increased sucrose synthase and sucrose phosphate synthase in cotyledons, thus high level of bound

fructose was found in cotyledons; whereas significant increment of reducing sugar in shoots after same hormone application may be due to induction of acid invertase (Kaur *et al.*, 2000). During germination of chickpea, cytokinins were first detected in the embryonic axes, which played important regulatory role in mobilizing main reserves from the cotyledon towards embryo proper (Villalobos and Martin, 1992). Exogenous application of other signal molecules like salicylic acid also enhanced α-amylase activity during seed development and germination in different pea varieties (Murtaza and Asghar, 2012). Besides phytohormones and growth regulators, animal derived components also exhibited wide range of bioactivity during germination and growth of chickpea seedlings. Exogenous application of mammalian steroidal sex hormones like progestesterone and androsterone significantly improved morphological, biochemical and antioxidant parameters of chickpea (Erdal *et al*, 2012). Conversely, plant based components like alkalase derived peptide hydrolysates of chickpea are pharmacologically active against angiotensin-I converting enzyme of mammalian system (Pedroche *et al.*, 2002). Antifungal peptides namely cicerin and arietin with N-terminal unique sequences were recently isolated from seeds of chickpea (Ye *et al.*, 2001). Defensin is another group of antimicrobial peptide isolated from chickpea and this peptide showed bioactivity against fungus *Pythium aphanidermatum* under wide pH range (Islam, 2008). But so far the physiological role of oligopeptides expressed or accumulated during post-germinating phases of chickpea seedlings are mostly unknown. In this study, peptides were isolated and partially purified from one week old chickpea seedlings and the bioassays were performed for the α-amylase induction, guard cell behaviour, chlorophyll retention and seedling vigour to determine the significance of peptide as a modulator of germination, growth and senescence.

6.2 MATERIALS AND METHODS

6.2.1 Plant culture

Seeds of dicotyledonous plant material, chickpea (Bengal Gram) [*Cicer arietinum* L.], collected from Central Pulses Research Institute (C.P.R.I.), Berhampur, West Bengal, India was weighed out (500g) and allowed to culture in laboratory conditions as specified in Chapter III Section 3.2.1.

6.2.2 Isolation and purification of low molecular weight peptides

The isolation and purification of peptides from 1 kg chickpea seedlings was performed according to the process mentioned in Chapter III Section 3.2.2.

6.2.3 Bioassay for induction of α-amylase release test

6.2.3a *Materials:*

A) Viable wheat (*Triticum aestivum* cv. *sonalika*) or Barley [*Hordeum vulgare* L.] cv. Narendra Barley-1 (NDB-209) [Dwarf variety] seeds

B) Different hours of germinating chickpea

6.2.3b *Enzyme Preparation and assay:*

Mentioned in details in Chapter III Section 3.2.8b

6.2.4 Stomatal aperture, opening and closing

Discussed in details in Chapter III Section 3.2.9

6.2.5 Bioassay for chlorophyll retention

3.2.5a *Plant cultivation and treatment:*

Seeds of mung bean were sown in well manure field and recommended doses of N, P_2O_5 and K_2O were applied before sowing. Different concentrations of hormones and peptides were applied twice till drenched, once at pre-flowering vegetative stage and second time at flowering reproductive stage. Spraying was done between 8 to 10 am of bright sunny days using 0.1% (v/v) Tween-20 as detergent. Altogether 14 treatments were given as foliar spray including two types of control – namely water spray and without spray.

6.2.5b *Estimation of chlorophyll and pigments:*

Different stages of mature leaves of chickpea [WAS *i.e.* weeks after sowing] were sprayed with specified peptide components or hormones with exact doses and the chlorophyll contents of leaves were determined by the method of Arnon (1949) and as modified by Kirk (1968). The same extract was measured at 480 nm, in spectrophotometer to estimate the carotene (Kirk and Allen, 1965). The methods were discussed in details in Chapter III Section 3.2.10b.

6.2.6 Determination of morphometric parameters

3.2.6a *Seed Priming:*

The seeds were sterilized by using 30% sodium hypochlorite for five minutes and then washed three times with distilled water. The seeds were then soaked in aerated solutions of all the treatments of peptides and hormones for six hours. A non-soaked, non-dried treatment was included as a control. After soaking, seeds were given three surface washings with sterilized water (Khan, 1992) and re-dried, near to original weight with forced air under shade. The seeds were then sealed in polythene bags and stored in refrigerator till further use (Basra *et al.*, 2003).

6.2.6b *Measurement of seedling vigour:*

Length and fresh weight (FW) of shoot and root were determined immediately after harvesting while dry weight (DW) was determined after drying these tissues at 80°C in an oven for 24 hours (Wahid *et al.*, 2008).

6.2.7 Bioassay guided purification of isolated peptides through Sephadex LH-20

The peptides obtained from ultrafiltration were fractionated through Sephadex LH-20 column (80 x 3) with ethanol (30%) fitted with ISCO fraction collector, UV-recorder and peristaltic pump (delivery 30 ml/h), and were collected in 200 tubes (5 ml in each tube) (Ghosh *et al.*, 2010). The tubes were grouped into 15 fractions according to spectral characteristics and ninhydrin response after discarding first 26 tubes. After grouping, all the fractions were lyophilized and suitably diluted for the purpose of bioassay.

6.3 RESULTS AND DISCUSSION

6.3.1 Amylase induction and mobilization of starch

During germination of legume seeds, growth and development of embryo proper is mainly dependent on mobilization of reserve food materials from cotyledons. Amylases are the particular enzymes responsible for conversion of complex polysaccharide into simple sugar for active absorption and uptake by embryo proper. As already stated gibberellins are the predominant hormones regulating induction and cellular targeting of α-amylase in germinating seeds in normal as well as in stressed condition (Kaur *et al.*, 2000). Besides GA_3, the role of steroidal sex hormones of mammalian origin (Erdal *et al.*, 2012) and salicylic acid (Murtaza and Asghar, 2012) were investigated in

Table 6.1 Amylase activity (nmoles of maltose generated min^{-1} g^{-1} dry matter) of untreated and germinated chickpea seeds soaked with GA_3 or peptides isolated from chick pea

Untreated	Treatment: GA_3 (M) or Peptide (ppm)		Soaked	Germinated (hours after soaking)						
				24 h	48 h	72 h	96 h	120 h	144 h	168 h
7.01 ± 0.8	Aqueous		8.57 ± 0.9	12.17 ± 2.3	18.71 ± 2.3	24.73 ± 2.4	32.72 ± 3.2	39.05 ± 2.7	42.14 ± 2.7	42.75 ± 1.8
	GA_3	10^{-3}	8.67 ± 1.2	14.22 ± 2.4	21.72 ± 2.5	28.14 ± 2.6	34.18 ± 3.3	40.02 ± 2.6	45.57 ± 2.9	46.06 ± 2.5
		10^{-4}	9.35 ± 1.4	17.43 ± 2.1	26.11 ± 2.3	31.65 ± 2.5	37.69 ± 3.1	44.56 ± 3.1	47.91 ± 3.1	48.98 ± 2.6
		10^{-5}	10.89 ± 1.1	20.63 ± 2.2	30.32 ± 2.6	36.71 ± 2.8	41.16 ± 3.0	48.93 ± 2.9	54.53 ± 3.4	56.71 ± 3.5
		10^{-6}	10.21 ± 1.3	19.12 ± 2.4	28.73 ± 2.7	33.91 ± 2.9	38.45 ± 2.9	45.97 ± 3.3	50.97 ± 3.1	52.28 ± 3.2
		10^{-7}	9.87 ± 1.2	16.35 ± 2.1	25.37 ± 2.8	30.88 ± 3.0	36.71 ± 2.8	43.67 ± 3.2	44.56 ± 2.8	46.71 ± 3.3
		10^{-8}	9.54 ± 1.5	15.61 ± 2.5	22.34 ± 2.7	25.67 ± 2.4	33.48 ± 2.6	42.26 ± 3.1	42.77 ± 2.7	44.93 ± 3.1
		10^{-9}	9.18 ± 1.1	13.38 ± 2.8	19.87 ± 1.8	22.97 ± 2.5	30.19 ± 2.5	38.38 ± 2.8	40.12 ± 2.6	40.97 ± 2.7
	Peptide	10	7.97 ± 1.3	11.38 ± 2.4	15.98 ± 1.9	20.37 ± 2.2	22.91 ± 2.7	32.37 ± 2.5	35.46 ± 2.4	36.11 ± 2.4
		1	8.22 ± 1.2	12.37 ± 2.5	17.61 ± 2.2	23.88 ± 2.1	31.28 ± 2.8	36.57 ± 2.6	37.29 ± 2.1	38.12 ± 2.3
		0.1	8.46 ± 1.3	15.68 ± 2.6	20.19 ± 2.8	29.43 ± 2.6	35.19 ± 2.7	39.83 ± 2.8	44.45 ± 2.5	48.11 ± 2.7
		0.01	8.93 ± 1.0	17.19 ± 1.2	21.35 ± 2.9	30.33 ± 3.1	34.22 ± 3.0	40.82 ± 3.2	45.67 ± 2.8	45.31 ± 2.6
		0.001	9.11 ± 0.9	16.35 ± 1.4	18.81 ± 1.1	25.19 ± 2.6	33.11 ± 2.2	38.17 ± 3.1	43.91 ± 2.4	44.91 ± 2.3
		0.0001	10.29 ± 1.1	14.55 ± 1.9	16.22 ± 1.7	24.88 ± 2.4	30.19 ± 2.4	36.71 ± 2.9	41.19 ± 2.2	40.81 ± 2.1

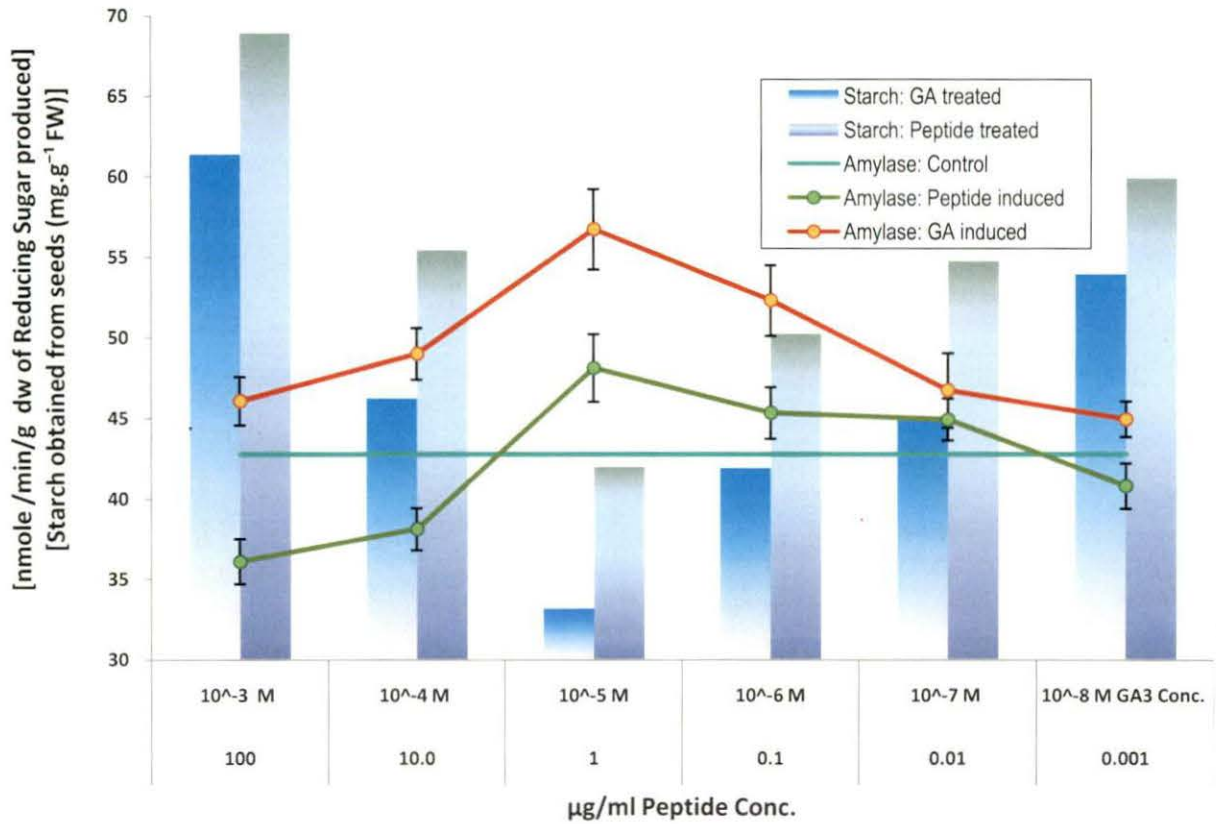

Figure 6.2 Amylase induction and relative starch content in wheat seeds by incubation with GA_3 and semi-pure peptides (3000-500 D) of chickpea

germinating chickpea and it was found that both the components improved the growth of embryo proper. In this study, the time dependent induction of total amylases were investigated in germinating chickpea seeds treated with low molecular weight (500-3000 Dalton) oligopeptides obtained after ultrafiltration along with GA_3 standard and control. Application of GA_3 exhibited best response at 10^{-5} (M) concentration and the highest amylase activity was observed after 168 hours of soaking [56.7 nmol maltose.min^{-1}.g^{-1} dry matters]; but when the induction pattern in respect to control was considered, maximum enhancement from control was noticed at 24 hours (1 day) [69.5%] of seed imbibitions (Table 6.1). Like GA_3, isolated peptides also induced amylase significantly at 24 hours when compared with control. Though peptide application improved amylase activity up to 168 hours (7 days) of post-imbibition phases, rate of enhancement in respect to control was gradually reduced with time after soaking. Peptides at concentrations between 0.1 to 0.01 ppm induced best response and the activity was enhanced up to 28.81% and 41.25% from control at 24 hours from soaking with 0.1 and 0.01 ppm respectively (Table 6.1).

For evaluation of amylase induction properties of isolated chickpea peptides in monocotyledons, the peptides were applied aseptically in embryoless half of wheat seeds and amylase activity of peptide treated seeds were monitored after 72 hours of incubation with control set. As it is already known that GA_3 is very responsive for amylase induction from aleurone layers of monocot seeds during germination, the same phytohormone was taken here as standard positive control. From Figure 6.2, it is apparent that GA_3 enhanced total amylase remarkably within 72 hours and the optimal hormonal responses were achieved at 10^{-5} (M) hormonal concentration [56.71 nmoles of reducing sugar produced / min / g dry weight of seeds]. Unlike GA_3, peptides at higher concentrations suppressed amylase synthesis as evidenced from inhibition of total amylase activity in wheat seeds, but at concentration 1 ppm or below, the same peptides partially induce amylase in embryoless half wheat seeds. Optimum induction was observed at 1 ppm peptide incubation where amylases from germinating wheat seeds produce 48.11 nmoles of reducing sugar / min / g dry weight, which is 12.54% higher than control (Figure 6.2). This induction was however not achieved by applying peptide concentrations lower than or equal to 0.001 ppm (Figure 6.2). For determining starch mobilization capacity, whole wheat seeds were incubated with peptides and after 3 days, residual starch content in wheat seeds were measured after removal of embryo proper. In all cases, it was observed that residual starch content was inversely related

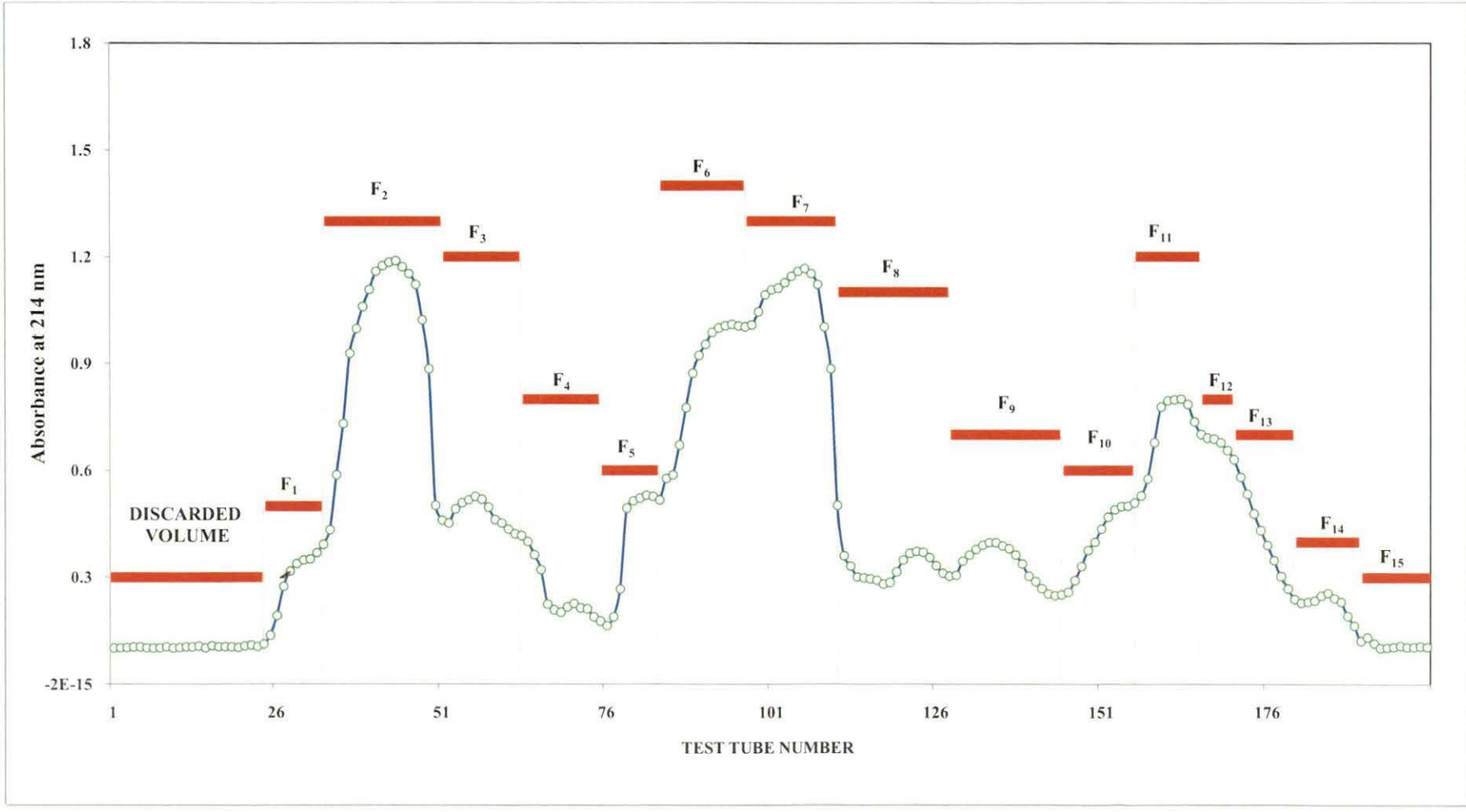

Figure 6.3 Peptide fractions after LH-20 purification and their absorbance pattern at 214 nm

with amylase induction pattern (Figure 6.2). Residual starch content was minimum, where the seeds were incubated with 10^{-5} (M) GA_3 or 1 ppm peptides (33.18 and 41.97 mg starch.g^{-1} FW for GA_3 and peptide respectively), which were their optimal doses for expression of amylases. The results indicated that peptides isolated from chickpea can able to induce amylases, at least partially in respect to GA_3 in other unrelated species like wheat. So the isolated peptides partly mimic the action of GA_3. Further it appears that the enhanced amylases by peptides are particularly utilized for degradation and/or mobilization of starch from storage organs during germination. Recent reports indicated that during salt stress, GA_3 can reverse the stress induced inhibition of germination and seedling growth. In chickpea, improved germination and seedling growth during stress condition was mediated through enhanced amylase activity and subsequent degradation of starch from cotyledons (Kaur *et al.*, 1998), as observed in our cases, where peptide mediated induction of amylases in germinating wheat seedlings were associated with retardation of starch content from endosperm.

As suggested by different authors, after ultra-filtration, gel exclusion and/or ion exchange chromatography may be the natural choice for achieving high degree purification of bioactive peptides from plants. Two antifungal peptides of 8.2 and 5.6 KDa were purified previously from seeds of chickpea through CM-Sepharose (Ye *et al.*, 2002). Earlier researchers also isolated angiotensin-I converting enzyme (ACE) inhibitory tripeptide Gly-Pro-Pro from buckwheat (*Fagopyrum esculentum* Moench) by passing protein extracts through 10 KDa YM-10 ultra-filtration membrane followed by further purification with ion exchange, LH-20 gel filtration chromatography and reverse phase HPLC (Ma *et al.*, 1997). In this study, after performing bioassays, ultrafiltered oligopeptides were further purified through Sephadex LH-20 gel exclusion chromatography. LH-20 columns were associated with peristaltic pump and the peptides were eluted with 30% ethanol and column eluted peptide fractions were distributed into 200 test tubes with the help of automated fraction collector, from which volumes occupied by first 26 test tubes were discarded. As already stated, all the naturally occurring hormones (IAA, GA_3, Zeatin and ABA) came within first 26 test tubes (130 ml per column volume of 565 ml), thus chances of existence of phytohormones during purification was minimized through removal of 130 ml solution of first 26 test tubes. Absorbance of each test tube was measured at 214 nm to determine the amount of peptides in each (Figure 6.3). According to their spectrophotometric responses at 214 nm wavelength, the test tubes were grouped into 15 fractions. From Figure 6.3, it can be

Table 6.2 Amylase induction in germinating wheat seeds by incubation with LH-20 purified fractions of semi-pure peptides (500-3000 Da) of chickpea

Peptide Fraction No.	**Amylase activity in different concentrations of peptides** [nmole of Reducing Sugar produced min^{-1} g^{-1} Dry Matter (Mean ± SD), with three replicates]					**Amylase induction with peptides** [Mean percentage increase/decrease over control]			
	Control	*Peptide concentrations*				*Peptide concentrations*			
		100 ppm	*10 ppm*	*1 ppm*	*0.1 ppm*	*100 ppm*	*10 ppm*	*1 ppm*	*0.1 ppm*
F_1	41.75 ± 1.8	38.21 ± 2.3	38.82 ± 1.9	39.29 ± 2.0	40.88 ± 2.2	-8.48	-7.02	-5.89	-2.08
F_2	41.87 ± 1.9	44.68 ± 2.1	45.77 ± 2.2	46.01 ± 2.4	45.87 ± 2.8	6.71	9.31	9.89	9.55
F_3	40.09 ± 2.1	39.23 ± 2.5	40.24 ± 1.8	41.88 ± 2.3	42.01 ± 2.5	-2.15	0.37	4.46	4.79
F_4	40.04 ± 1.4	37.81 ± 1.6	36.67 ± 1.5	36.08 ± 1.6	37.22 ± 1.8	-5.57	-8.42	-9.89	-7.04
F_5	43.88 ± 1.1	40.98 ± 1.7	41.77 ± 2.3	43.97 ± 2.7	42.28 ± 2.3	-6.61	-4.81	0.21	-3.65
F_6	44.21 ± 1.6	43.89 ± 1.9	44.25 ± 2.4	46.87 ± 2.7	47. 93 ± 3.1	-0.72	0.09	6.02	8.41
F_7	43.76 ± 2.2	44.67 ± 2.6	45.22 ± 2.2	45.96 ± 2.8	44.13 ± 2.1	2.08	3.34	5.03	0.85
F_8	41.19 ± 2.5	39.11 ± 2.3	38.76 ± 1.4	39.01 ± 1.7	39.98 ± 1.6	-5.05	-5.90	-5.29	-2.94
F_9	43.99 ± 2.3	40.23 ± 1.5	41.17 ± 2.5	42.86 ± 2.4	43 19 ± 2.9	-8.55	-6.41	-2.57	-1.82
F_{10}	41.16 ± 2.5	38.89 ± 1.8	38.21 ± 1.9	40.66 ± 2.4	42. 35 ± 2.5	-5.52	-7.17	-1.21	2.89
F_{11}	42.23 ± 2.3	40.69 ± 2.4	41.26 ± 2.7	42.33 ± 2.4	44.15 ± 2.8	-3.65	-2.30	0.24	4.55
F_{12}	40.88 ± 1.5	40.98 ± 1.8	41.24 ± 2.3	42.47 ± 2.5	39.17 ± 2.4	0.24	0.88	3.89	-4.18
F_{13}	44.02 ± 1.7	41.86 ± 2.4	42.17 ± 2.5	43.52 ± 2.7	42 29 ± 2.9	-4.91	-4.20	-1.14	-3.93
F_{14}	43.01 ± 1.6	39.08 ± 2.6	40.61 ± 1.9	41.22 ± 2.3	42.57 ± 2.8	-9.14	-5.58	-4.16	-1.02
F_{15}	41.28 ± 1.8	39.01 ± 2.3	40.44 ± 2.4	41.56 ± 2.6	43.91 ± 2.1	-5.50	-2.03	0.68	6.37
GA_3 Standard	42.23 ± 1.9	46.78 ± 2.3	68.75 ± 3.3	57.98 ± 2.8	52.18 ± 2.4	20.25	62.80	37.30	23.56

stated that F_2, F_6, F_7 and F_{11} contain significant amount of peptides, when compared with other fractions of chickpea peptides. Bioactivity (amylase induction) of those peptide fractions in different concentrations based on their fresh weight was determined by measuring amylase activity in embryo-less half wheat seeds after 72 hours incubation with peptides. Table 6.2 shows concentration dependent amylase activity in terms of nano-moles of reducing sugars produced per minute per gram dry weight of seeds incubated with different LH-20 peptide fractions and also the percentage of induction or inhibition of amylases along with GA_3 standard. Amylase activity was best realized in seeds incubated with peptide fraction F_2 and F_6 at their optimal dose of 1μg/ml and 100ng/ml respectively. Amylase induction may be from 9.3% to 9.9% for F_2 fractions with applied peptide doses ranges between 10 to 0.1 ppm, whereas the same may be from 6% to 8% with 1.0 or 0.1 ppm peptide incubation (Table 6.2). Inhibition of amylase activity was also watched in peptide fractions F_1, F_5, F_9, F_{10} and F_{14}. Inhibition was realized mostly when concentrated doses of peptides (100 ppm) were applied on germinating wheat seeds and highest level of inhibition was detected (9.14%), when F_{14} peptide fraction was applied at 100 ppm dose (Table 6.2). Previously angiotensin-I converting enzyme inhibitory peptides from *Fagopyrum esculentum* were separated from other proteins by passing through YM-10 (10,000 Da cut-off) ultra-filtration membrane and further bioassay guided purification was achieved by using successive chromatographic methods involving ion exchange, gel filtration with Sephadex LH-20 and reverse phase HPLC (Ma *et al.*, 1997). But in this study the restoration of bioactivity after LH-20 purification was not very significant (maximum 9.89% induction from control). So, further consecutive chromatographic methods were not performed for ultimate purification of peptides.

6.3.2 Retention of chlorophyll

In leguminous plants, cytokinins are the main group of phytohormones participate in the essential aspects of leaf senescence (Anil *et al.*, 1985). But evidences suggest that single plant hormone may not be functionally responsive in delaying or initiating senescence process (Joyce and Thomas, 1980). Leaf senescence in *Cicer arietinum* appears to be significantly correlated with the degradation of essential protein and chlorophyll content as well as the reduced assimilation of sugars in leaves (Ali and Bano, 2008). Earlier investigations suggested that the foliar application of kinetin delay leaf senescence (Hajouj *et al.*, 2000), whereas ABA was more effective in accelerating the same (Samet

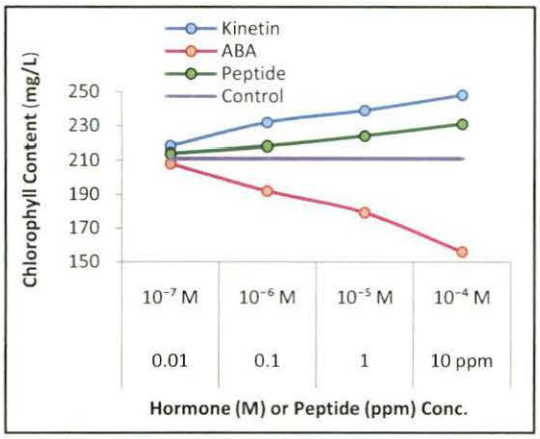

Figure 6.4 Chlorophyll retention in young leaves at 8 WAS

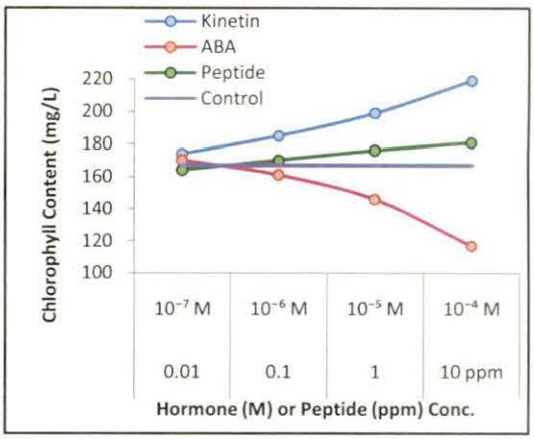

Figure 6.5 Chlorophyll retention in young leaves at 16 WAS

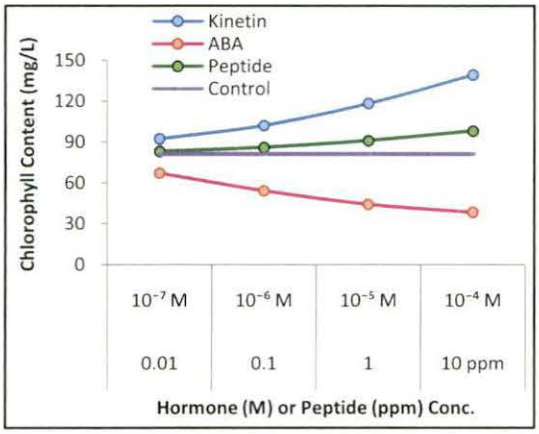

Figure 6.6 Chlorophyll retention in young leaves at 20 WAS

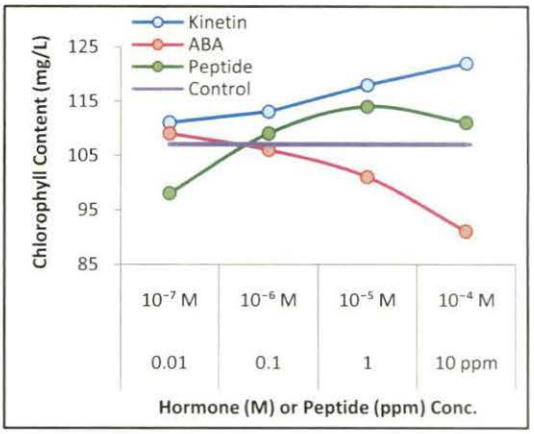

Figure 6.7 Chlorophyll retention in mature leaves at 8 WAS

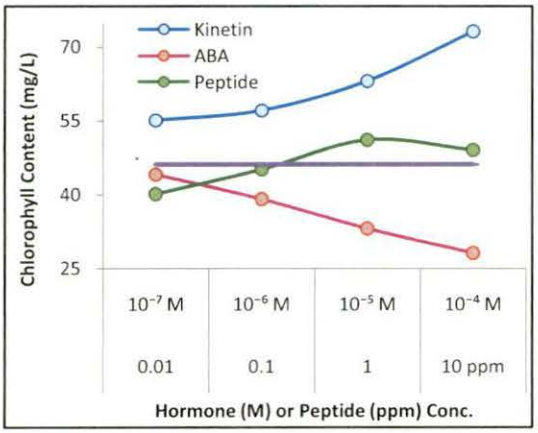

Figure 6.8 Chlorophyll retention in mature leaves at 16 WAS

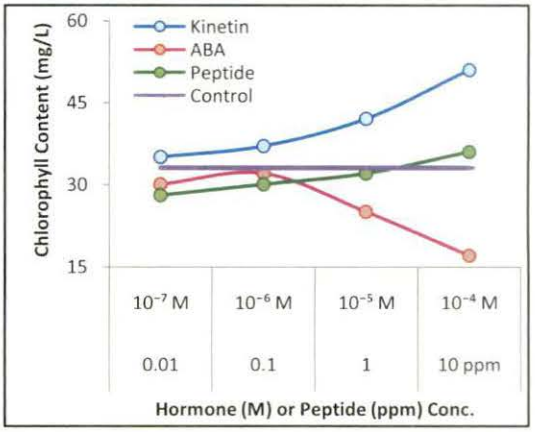

Figure 6.9 Chlorophyll retention in mature leaves at 20 WAS

Table 6.3 Chlorophyll retention in mature leaves of *Cicer arietinum* L. [10 WAS] after application of LH-20 purified peptides and benzyl adenine (BA)

Samples	*Total Chlorophyll Content (mgL^{-1})*				*Mean % of chlorophyll retention*			
Initial State	243 ± 12				-			
Control	108 ± 05				0.00			
BA (10^{-5} M)	129 ± 09				19.44			
Peptide Fraction No.	*Peptide concentrations*				*Peptide concentrations*			
	100 ppm	*10 ppm*	*1 ppm*	*0.1 ppm*	*100 ppm*	*10 ppm*	*1.0 ppm*	*0.1 ppm*
F1	101 ± 04	105 ± 06	111 ± 07	104 ± 05	-6.48	-2.78	2.78	-3.70
F2	114 ± 07	116 ± 08	112 ± 11	109 ± 06	5.56	7.41	3.70	0.93
F3	109 ± 08	108 ± 05	106 ± 08	98 ± 07	0.93	0.00	-1.85	-9.26
F4	99 ± 05	98 ± 06	97 ± 04	101 ± 12	-8.33	-9.26	-10.19	-6.48
F5	113 ± 10	106 ± 09	107 ± 06	103 ± 03	4.63	-1.85	-0.93	-4.63
F6	117 ± 05	115 ± 04	113 ± 06	105 ± 06	8.33	6.48	4.63	-2.78
F7	118 ± 12	112 ± 09	108 ± 07	102 ± 03	9.26	3.70	0.00	-5.56
F8	109 ± 05	105 ± 06	101 ± 03	100 ± 04	0.93	-2.78	-6.48	-7.41
F9	111 ± 13	107 ± 06	105 ± 05	109 ± 07	2.78	-0.93	-2.78	0.93
F10	112 ± 11	103 ± 05	106 ± 08	101 ± 06	3.70	-4.63	-1.85	-6.48
F11	114 ± 07	116 ± 07	119 ±12	108 ± 05	5.56	7.41	10.19	0.00
F12	115 ± 11	118 ± 14	112 ±09	105 ± 06	6.48	9.26	3.70	-2.78
F13	109 ± 10	103 ± 04	101 ± 05	95 ± 05	0.93	-4.63	-6.48	-11.11
F14	110 ± 05	106 ± 07	102 ± 01	98 ± 04	1.85	-1.85	-5.56	-9.26
F15	104 ± 05	100 ± 04	107 ± 06	112 ± 13	-3.70	-7.41	-0.93	3.70

and Sinclair, 1980). ABA application significantly decreased leaf chlorophyll content and the mature leaves are more susceptible to ABA than their younger counterparts (Chaloupkova and Smart, 1994). Also leaf senescence in chickpea and their influence with the application of phytohormones like kinetin and ABA is associated with growth and development of plants (Ali and Bano, 2008). As the different growth stages of plant and maturity of leaves had significant impact on chlorophyll degradation, the effect of isolated peptides on chlorophyll retention were evaluated at different growth stages, measured in weeks after sowing (WAS): vegetative (8 WAS), flowering (16 WAS), and pod filling stages (20 WAS) in young and mature leaves. In all cases, Kinetin and ABA was adjusted as positive and negative control respectively and the effect on foliar application of these hormones on total chlorophyll content was determined separately. In both young and mature leaves, percentage of chlorophyll retention was enhanced by the application of kinetin but the retention capacity was improved [72%] during pod filling stage [20 WAS] in young leaves, whereas the same phytohormone was more responsive at 16 WAS [flowering stage] in case of mature leaves [58% more retention of chlorophyll in respect to control] (Figure 6.4-6.9). ABA on the other hand, accelerate chlorophyll degradation after the commencement of reproductive stages in young leaves, while in mature leaves, the same was accelerated with the approach of growth stages (Figure 6.4-6.9). Interestingly, in young leaves peptides retard chlorophyll degradation in respect to control significantly during pod filling stages (21% improved) but the response was appreciably lower than kinetin (Figure 6.4-6.6). When the same peptides was applied on mature leaves, chlorophyll retention capacity was improved at 1 ppm concentration during flowering stages (16 WAS), but here also the activity was much inferior to kinetin action (Figure 6.7-6.9).

After Sephadex LH-20 purification, chlorophyll retention was again performed with different peptide fractions in mature leaves of *Cicer arietinum* during their vegetative stages of growth [10 WAS]. Here benzyl adenine [BA: 10^{-5} (M)] was taken as standard and percentage of chlorophyll retention with foliar application of BA in respect to control was observed as 19.44% (Table 6.3). In case of peptides, relatively high chlorophyll retention was observed in F_6, F_7, F_{11} and F_{12} LH-20 fractions when applied on mature leaves, though the bioactivity was much lower than BA (10^{-5} M). Among bioactive fractions, 100 ppm or higher concentrations were required for F_6 and F_7 for attainment of significant retardation of chlorophyll loss (8-9%) by peptide treatment, and in case of F_{11} and F_{12}, optimized chlorophyll retention was observed, if

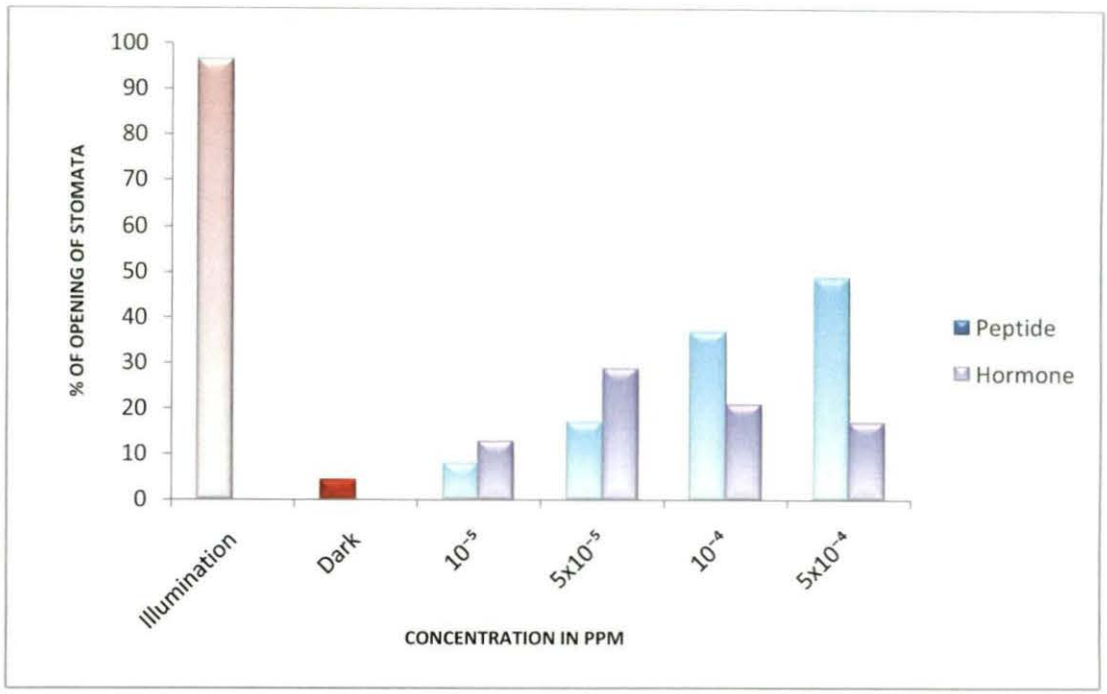

Figure 6.10 Percentage of stomatal opening with ultrafiltered peptides (0.5-3.0 KDa) and Benzyl adenine in dark

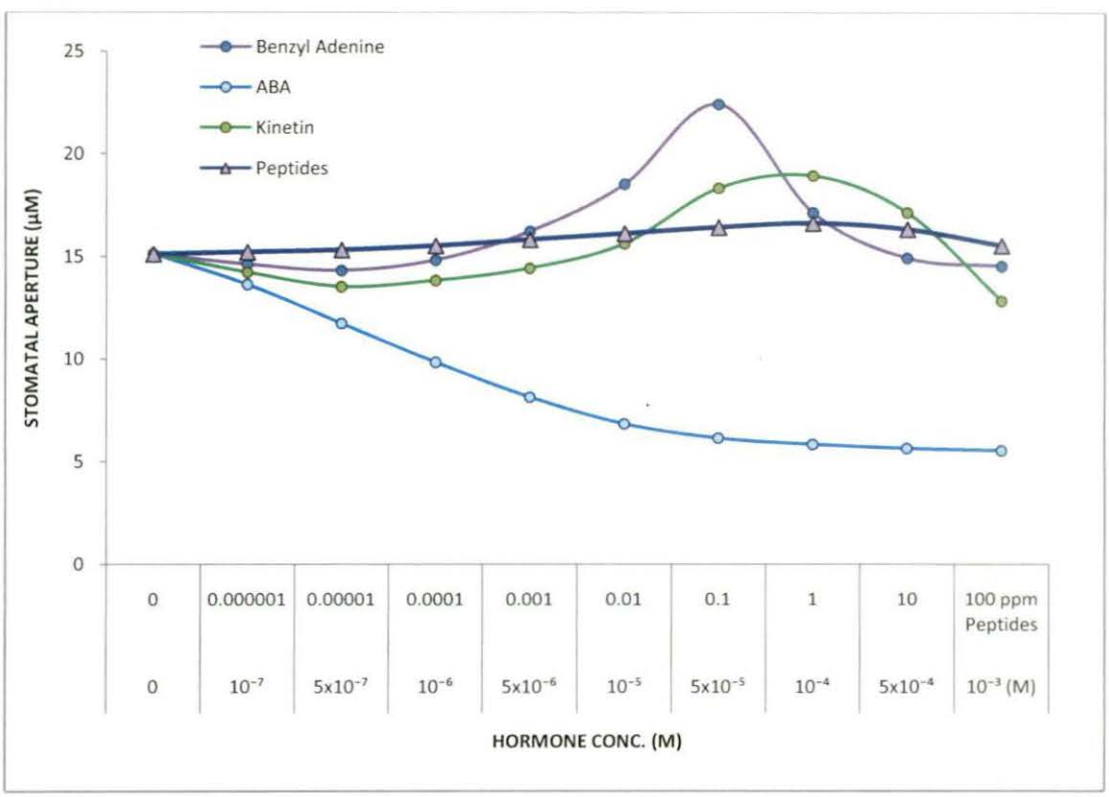

Figure 6.11 Effect of peptides and hormones on stomatal aperture control after illumination in *Commelina benghalensis*

applied in between 1 to 10 ppm doses (Table 6.3). Some fractions like F_1, F_4, F_{10} and F_{13} also accelerate chlorophyll loss from mature leaves significantly, if incubated at their optimized doses. Overall, it may be stated that peptide treatment didn't improve chlorophyll retention remarkably from control even after purification through LH-20 gel filtration column. In this scenario, further bioassay guided purification of peptides isolated from *Cicer arietinum* were not carried out, as the productive yield of peptides in terms of bioactivity (i.e. considerable chlorophyll retention capacity) was not obtained from first column fractions.

6.3.3 Stomatal guard cell regulation

Considerable attention has been receiving in recent decades on stomatal aperture control and the rate of transpiration through exogenous application of hormones and other phytochemicals. By closing or opening of stomata, the guard cells regulate transpiration rate for controlling water retention or loss and maintaining homeostatic balance of water in plant body (Zhang *et al.*, 2001). Abscisic acid (ABA) significantly reduced the aperture width of stomata when applied on leaves and thus the hormone was considered to be as antitranspirant (Talha and Larsen, 1975). Conversely, stomatal opening and guard cell aperture was increased by the application of cytokinins (Das *et al.*, 1976). Stomatal conductance and guard cell aperture was also affected by different light treatments. Clear decrease in stomatal conductance was observed in the dark as reflected from data obtained by porometer in *Vicia faba* leaves (Blom-Zandstra *et al.*, 1995). On the other hand, sunlight induces stomatal opening and aperture width of guard cells significantly in different herbaceous plants (Knapp and Smith, 2006). In this experiment, after ultrafiltration bioactivity of isolated peptides (0.5-3.0 KDa) from chickpea on stomatal guard cell response was evaluated under light and dark treatment. For determining stomatal behaviour, chickpea peptides were applied in other unrelated plant species *Commelina benghalensis*. Hormones like kinetin and benzyl adenine were taken as standard for measuring the degree of opening of stomatal aperture, whereas the ABA was taken as an inhibitor of guard cell movement. In untreated leaves of *Commelina benghalensis*, stomatal opening was 96.3% and 4.2% under illuminated and dark condition (Figure 6.10). In dark, benzyl adenine performs best response at $5x10^{-5}$ ppm concentration where stomatal opening was 29% (approximately 25% higher than control) (Figure 6.10). Stomatal opening response with peptide application was dose dependent in dark, i.e. better opening of stomata was observed with higher doses

Table 6.4 Stomatal Opening in *Commelina benghalensis* after application of chick pea peptides in dark

Peptide Fraction No./ Hormone Standard	Percentage of stomatal opening in different concentrations of peptides				Width of stomatal aperture in different concentrations of peptides			
	Control	1 µg / ml	1 x 10^{-2} µg /ml	1 x 10^{-4} µg /ml	Range of aperture width (µm)	Mean value with standard deviation (µm)		
						1 µg / ml	1 x 10^{-2} µg /ml	1 x 10^{-4} µg /ml
Benzyl Adenine	3.8		32.7		14.2-18.5		15.8 ± 2.6	
F1	3.5	5.6	4.7	4.3	3.1-8.7	4.6 ± 1.6	5.4 ± 1.8	6.2 ± 2.2
F2	4.1	8.9	9.7	10.2	2.8-8.9	5.2 ± 2.4	6.1 ± 2.1	6.8 ± 2.3
F3	3.8	3.5	4.1	4.9	1.4-7.7	3.1 ± 2.1	3.5 ± 1.6	5.7 ± 1.9
F4	4.2	3.2	3.5	3.1	2.1-7.8	3.6 ± 1.6	3.8 ± 1.9	5.5 ± 2.3
F5	2.9	6.6	7.9	9.2	1.5-9.9	4.0 ± 2.5	6.1 ± 1.8	6.8 ± 3.1
F6	3.3	**14.8**	**18.6**	**19.7**	**3.7-12.1**	**7.1 ± 3.6**	**7.6 ± 3.3**	**8.8 ± 3.2**
F7	4.4	**13.2**	**15.5**	**21.6**	**4.7-14.4**	**7.8 ± 3.1**	**8.1 ± 3.6**	**10.5 ± 3.9**
F8	3.7	1.2	1.8	2.1	1.1-3.9	2.1 ± 1.1	2.3 ± 1.6	2.7 ± 1.2
F9	3.9	2.4	2.7	2.9	1.5-6.1	2.8 ± 1.3	3.7 ± 1.7	3.9 ± 2.2
F10	3.0	3.1	3.8	4.7	0.9-6.4	2.5 ± 1.9	3.2 ± 2.6	3.9 ± 2.6
F11	4.1	7.4	9.8	10.1	1.1-11.9	4.4 ± 3.6	7.5 ± 3.9	8.8 ± 3.2
F12	3.5	4.5	4.1	3.4	1.5-8.4	3.4 ± 1.9	4.9 ± 2.3	5.5 ± 2.7
F13	4.3	2.1	2.8	3.3	0.8-6.4	2.5 ± 1.8	3.9 ± 2.1	4.6 ± 2.8
F14	2.8	2.7	3.4	3.6	0.7-7.5	1.8 ± 1.1	3.7 ± 1.7	4.8 ± 2.8
F15	3.4	3.5	3.8	4.5	1.0-6.3	3.5 ± 2.5	4.5 ± 2.3	4.2 ± 2.1

of peptide treatment. Remarkably, oligopeptides induced stomatal opening up to 49% with maximum higher applied doses, and the response was even better than optimal doses of cytokinins (Figure 6.10). Under illumination, stomatal apertures of *Commelina benghalensis* leaves were measured under different hormone and peptide treatments. Maximum opening of stomatal aperture (22.4 μm) was observed with benzyl adenine application at 5 x 10^{-5} (M) concentration (Figure 6.11). Response of kinetin was better (stomatal aperture ranges between 17 to 19 μm) when applied between 5 x 10^{-4} to 5 x 10^{-5} (M) concentrations. Conversely, ABA inhibited the opening of stomatal aperture particularly when applied above 1.0 μM doses (Figure 6.11). Peptides of chickpea, on the other hand, induce opening of stomatal aperture only slightly (16.6 μm, i.e. 9% more than control) in illuminated leaf epidermal cells of *Commelina benghalensis* (Figure 6.11).

Stomatal opening in *Commelina benghalensis* leaves in dark was also measured after purification of peptides through LH-20 Sephadex gel column chromatography. In dark, only 3.8% stomata were opened in untreated (control) epidermal peels (Table 6.4). Benzyl adenine [applied concentration: 5 x 10^{-5} (M)], which was used as positive standard, opened stomata about 32.7% with guard cell aperture width ranges between 14.2 to 18.5 μm. Stomatal opening and guard cell aperture width was also evaluated with the treatment of fifteen different fraction of peptides obtained from LH-20 column at three specified concentrations (Table 6.4). When tested with different fraction of peptides, stomatal opening response was best observed with F_6 and F_7 peptides of *Cicer arietinum* on epidermal peels. Maximum stomatal opening of 19.7% and 21.6% was achieved by F_6 and F_7 peptide treatment with mean aperture width of 8.8 and 10.5 μm respectively at 100 picogram.ml^{-1} applied doses (Table 6.4). Though the induction of stomatal guard cell movement in dark by F_6 and F_7 peptide was significantly higher (4.9 times) than untreated control, the response was much lower than benzyl adenine (8.6 times higher than control). Other fractions when tested on epidermal peels in dark, didn't exhibit significant deviation from control; so these fractions may be considered as biologically inactive. As the amount of peptides obtained from bioactive fraction F_6 and F_7 was quite lower (<10 mg), further purification with consecutive chromatographic columns were not continued.

6.3.4 Improvement of seedling vigour after priming

It was already established that some plant growth regulators like gibberellins play a central role in the integration of cellular metabolic processes during germination, thus enhancing the rate of emergence and seedling growth (Kaur *et al.*, 1998). Pre-sowing treatment, also known as seed priming generates high impact osmo-conditioning effect, which improves seed performance, synchronized germination and seedling vigour (Siveritepe and Dourado, 1995). The beneficial impact of priming of seeds with different growth regulators have already been successfully utilized in many crop plants like sunflower (Kaya *et al.*, 2006), rice (Habib *et al.*, 2010), maize (Nawaz and Ashraf, 2010), cotton (Casenave and Toselli, 2007) and mustard (Srinivasan *et al.*, 1999). Also the application of plant growth regulators for improving seedling performance is well documented in many vegetables like pepper (Jeong *et al.*, 1994) and high value tree species, for example *Myrica esculenta* (Bhatt *et al.*, 2000). Among phytohormones, gibberellins were reported to be an excellent priming agent (Hay and Pederson, 1986). Hence, in this investigation, GA_3 was applied as standard priming agent for determining the priming potential of chickpea peptides for improving overall seedling vigour. Priming for 12 hours with GA_3 enhanced root and shoot length for about 15.63% and 76.92% respectively in germinating chickpea seedlings, when applied at optimal dose of 10^{-6} (M). Not only that, GA_3 at 10^{-5} (M) optimized priming treatment for same duration improved fresh biomass of seedlings up to 43.49%. Interestingly, besides conventional phytohormones, priming with vitamin (ascorbic acid) and salicylic acid also improved root and shoot length, seedling dry biomass and number of secondary roots in many crop species, when compared with untreated control (Khan *et al.*, 2011). For improving seedling morphology and vigour index of chickpea, 1% aqueous oxygenated peptone solution was also used by some authors as pre-sowing soaking treatment (Thakare *et al.*, 2006). Among nitrogenous compounds, seed priming with polyamines like putrescine, spermine and spermidine also promoted different observed attributes of seed germination and early seedling growth in *Capsicum annum* such as root and shoot enlargement, seedling fresh and dry weight, vigour index etc. (Khan *et al.*, 2012). Several studies have also shown the significant positive impacts of other different non-conventional priming agents on seed germination, seedling vigour and development (Afzal *et al.*, 2009). But till now almost no reports are available on application of bioactive peptides for improvement of seedling morphology through priming. In this study the effects of pre-sowing soaking treatment with different concentration of

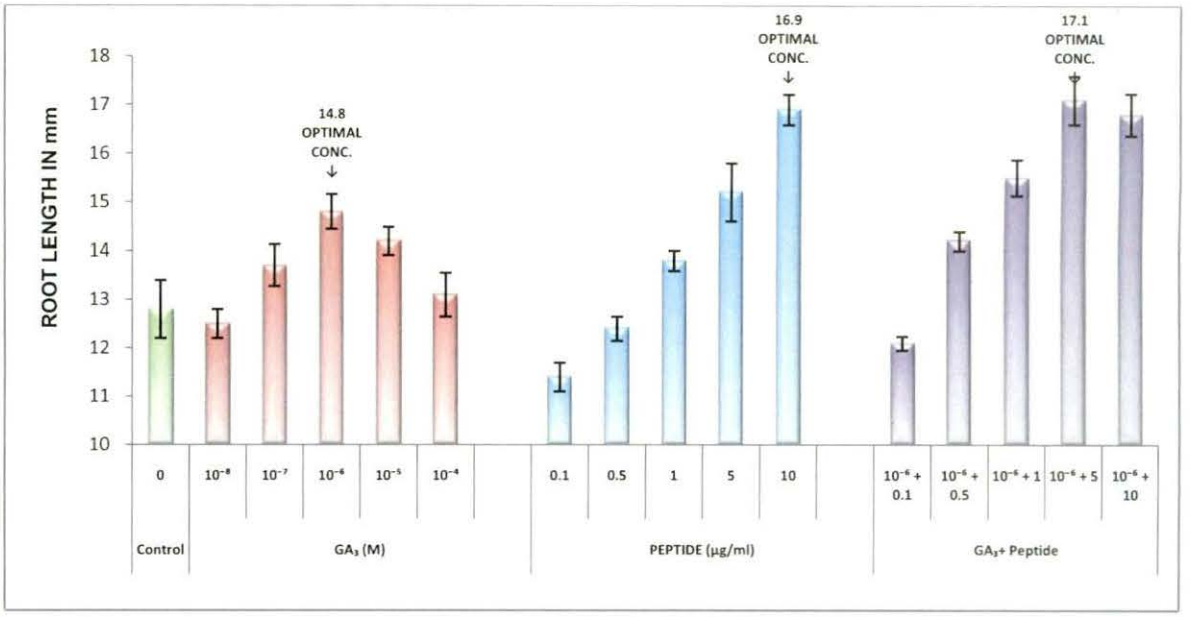

Figure 6.12 Effect of GA_3 and peptide priming on root length of germinating chickpea seedlings

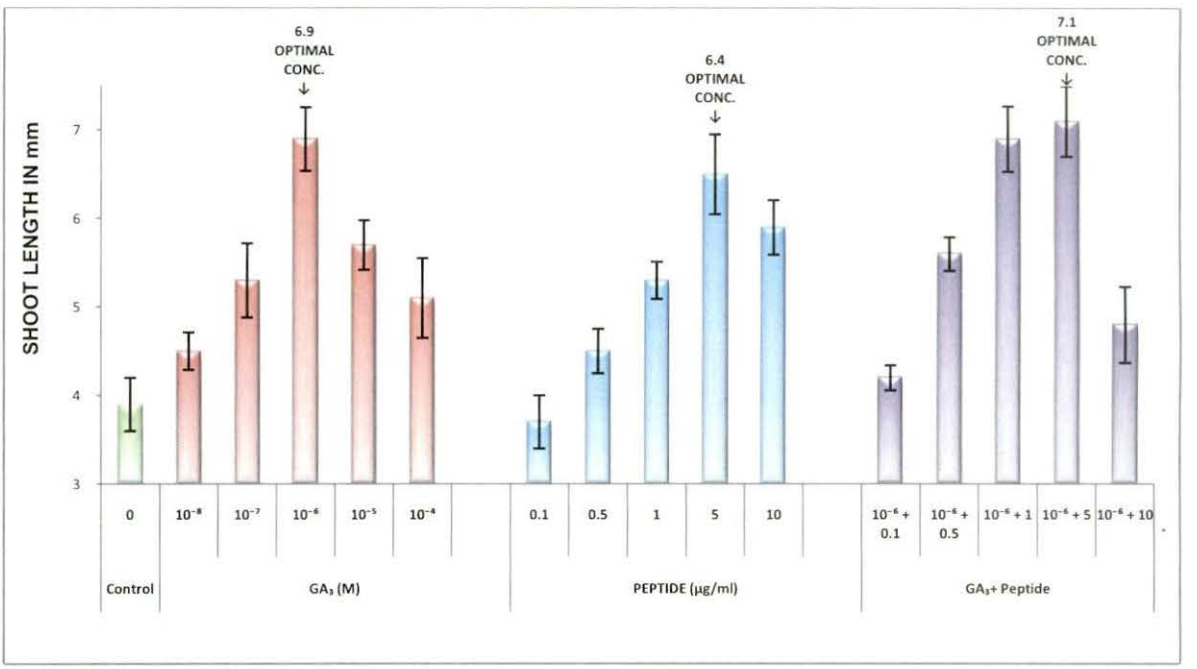

Figure 6.13 Effect of GA_3 and peptide priming on shoot length of germinating chickpea seedlings

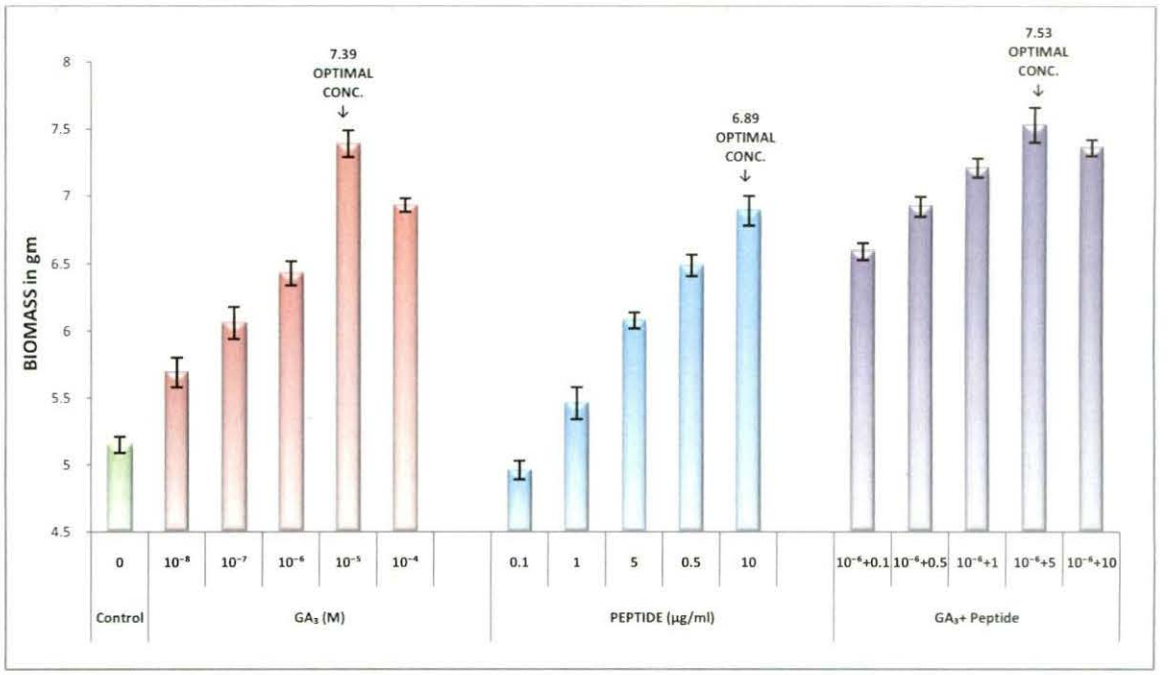

Figure 6.14 Effect of GA_3 and peptide priming on fresh biomass of germinating chickpea seedlings

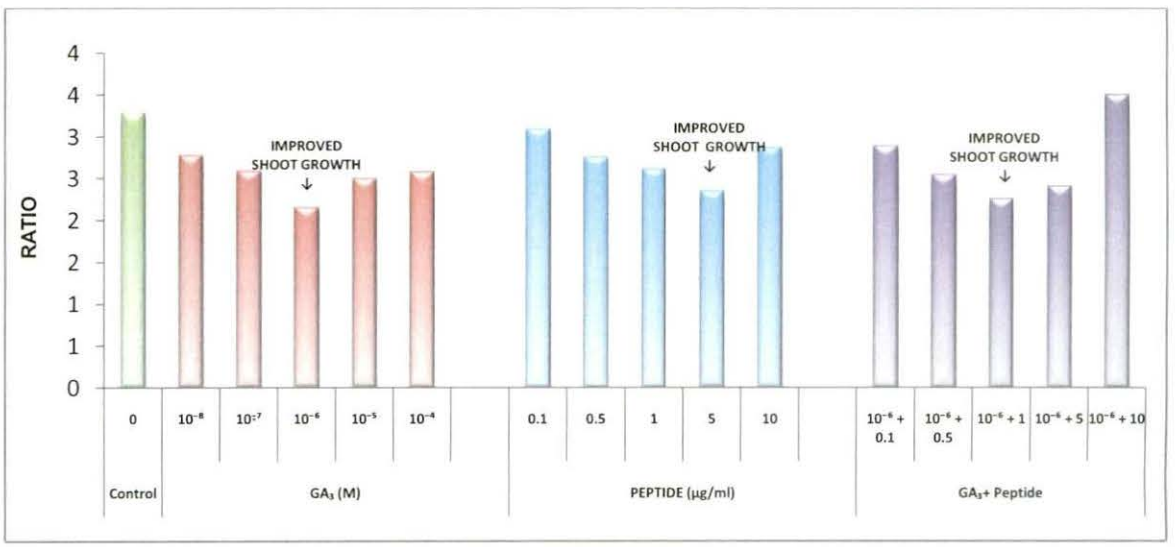

Figure 6.15 Effect of GA_3 and peptide priming on root vs. shoot ratio of germinating chickpea seedlings

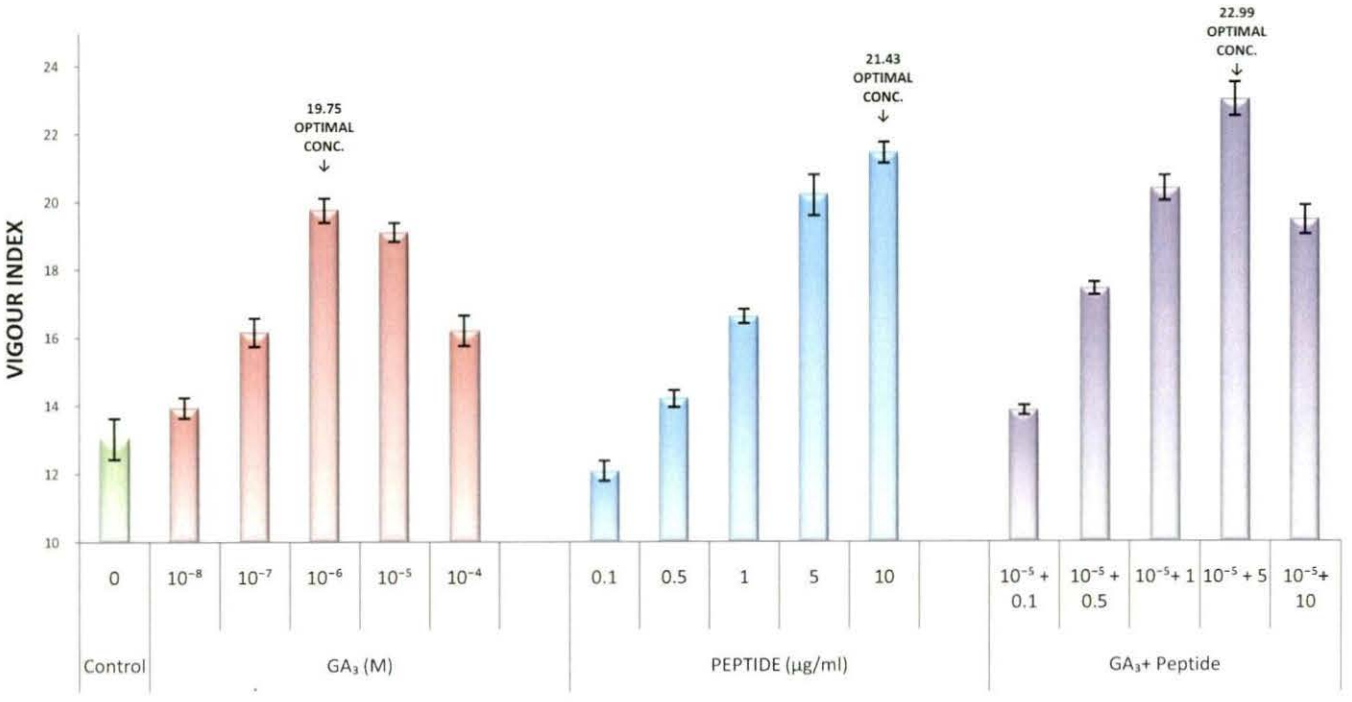

Figure 6.16 Effect of GA_3 and peptide priming vigour index of germinating chickpea seedlings

isolated heterogeneous oligopeptides (0.5-3.0 KDa) were determined on morphological parameters of germinating chickpea (6 DAS). From Figure 6.12, it may be stated that optimal root elongation of 16.8 mm was observed with at 10 ppm priming dose, and the improvement of growth was even far better than pre-sowing soaking treatment with optimal dose of GA_3 (15.63% enhancement). From Figure 6.13, it is also prominent that priming with peptides also improved shoot length significantly and at optimal dose of 5 ppm, shoot length attained 6.4 mm which is 66.67% higher than control, but the result was not so impressive like GA_3 (76.92% improvement). When overall fresh biomass is considered, pre-sowing soaking treatment with peptides at 10 ppm optimal dose enhanced fresh biomass up to 6.89g per seedling which is 33.79% greater than control (Figure 6.14). Here also the performance of GA_3 was superior (43.49% higher than untreated) than isolated peptides. In a nutshell, priming induced the enlargement of shoot in a prioritized manner as reflected from root shoot ratio of pre-soaked seedlings in Figure 6.15. When seedling vigour index is considered, optimum vigour was achieved by GA_3 and peptide priming at 10^{-6} and 10 ppm concentration respectively with improvement of 51.57% and 64.47% respectively from control (Figure 6.16). As pre-sowing soaking treatments with GA_3 and peptides improved seedling morphology significantly from control, during priming application of optimized dose of GA_3 and isolated peptides with varying concentrations were also given simultaneously for determining the synergistic activity of both the components in a combined form. But combined application of GA_3 and peptide as priming agent on chickpea seedlings didn't synergistically improve any of the morphological attributes of development (Figure 11-15). However combined application of these components as pre-sowing soaking agent significantly enhanced root (Figure 6.12) and shoot length (Figure 6.13), seedling biomass (Figure 6.14) and vigour index (Figure 6.16) at peptide concentrations ranged principally between 1 to 10 ppm, when compared with control. Recent molecular analyses documented that CLE group of oligopeptides in association with FCP1 protein functionally impair root and shoot apical meristem development and strongly inhibit different morphological attributes of germinating seedlings in different leguminous plant like *Medicago truncatula* (Oelkers *et al.*, 2008). In this scenario, priming with germination induced oligopeptides in chickpea may somehow degrade or prohibit the functional expression of inhibitory factors, thus upgrading the developmental attributes of embryo proper and mimicking the role of gibberellins during germination.

Table 6.5 Summary of bioactivity of ultra-filtered and column chromatographic fractions of different peptides isolated from seven days old *Cicer arietinum* L. (Bengal Gram) seedlings and their responses against different experiments. Sephadex LH-20 column (80 x 3 cm), volume – 565 ml approximately, eluted with 30% aqueous ethanol, and fractionated with 5 ml tube with pump speed – 30 ml/h. Tube number 1-24 or 120 ml is void volume approximately and any bioactivity, if found is rejected, and the rest 176 tubes were collected and screened for bioactivity

Fraction Tube Number:			**00-24**	**25-33**	**34-51**	**52-63**	**64-75**	**76-84**	**85-97**	**98-111**	**112-128**	**129-145**	**146-156**	**157-166**	**167-171**	**172-180**	**181-190**	**191-200**
Joined Fraction Number:			**VOID**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**
UV Absorbance:		214 nm	-	+	++++	+	-	+	+++	++++	-	-	+	++	++	+	-	-
		260 nm	-	-	+	-	-	-	+	++	-	-	+	+	+	-	-	-
		280 nm	-	+	++	+	-	-	++	++	-	-	+	++	++	-	-	-
0.2% Ninhydrin response:			-	-	+++	+	-	-	++	+++	-	-	+	++	+	-	-	-
α-Amylase elicitation:	Induction:		N/A	-	++	+	-	-	+	+	-	-	-	-	-	-	-	-
	Inhibition:		N/A	+	-	-	+	-	-	-	+	+	+	-	-	-	+	-
Control of stomatal guard cell:	Percentage of opening in dark		N/A	-	+	-	-	+	++	++	-	-	-	+	-	-	-	-
	Diameter of stomatal aperture		N/A	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-
	Percentage of closing in light:		No significant stomatal closure was observed in any fraction															
Retention of chlorophyll:			N/A	-	+	-	-	-	+	+	-	-	-	++	+	-	-	-
Growth of coleoptiles:	Elongation:		This bioassay was not performed with *Cicer arietinum* peptides															
	Inhibition:																	
Seedling Growth Response:	Root:		Root length increased up to **32.03** % above control at 10 ppm optimal crude peptide (ultrafiltered) concentration, not performed with LH-20 peptide fractions.															
	Shoot:		Shoot length increased up to **66.67** % above control at 5 ppm optimal crude peptide (ultrafiltered) concentration, not performed with LH-20 peptide fractions.															
	Seedling Biomass:	Fresh Weight:	Fresh biomass increased up to **33.79** % above control at 10 ppm optimal crude peptide (ultrafiltered) concentration, not performed with LH-20 peptide fractions.															
		Dry Weight:	NOT MEASURED															
Further purification with HPLC:			NOT PERFORMED															
Amino acid analysis of bioactive peptides:			NOT PERFORMED															
Sequencing of bioactive peptides:			NOT PERFORMED															

The physico-chemical characterization and the relative strength of different attributes of bioactivity of Sephadex LH-20 purified peptide fractions of *Cicer arietinum* were represented in Table 6.5. UV-absorbance and the ninhydrin response were better observed in F_2, F_6, and F_7 indicating that the peptides were accumulated in these fractions with high quantity. When the profile of bioactivity was concerned, most of the attributes exhibited promotive effect, associated with germination, growth and development of seedlings. Amylase induction was most prominently performed by peptides of F_2, whereas the dark mediated opening of stomata was observed by peptides present in F_6 and F_7. Again chlorophyll retention was partially exhibited by peptides of F_{11} fraction. Possibly heterogeneous group of peptides were distributed in different LH-20 fractions and performing a wide array of bioactivity related with germination and improvement of seedling growth. Most probably these peptides are proteolytically processed and secreted from cellular compartments and contribute in different functional physiology of germination either independently or in association with hormones. Different oligopeptide transporters were already characterized in germinating seeds and embryo proper of different crop species but the exact role of these transporter were yet known (West *et al.*, 1998). Presumably the transported peptides escalate different vital processes associated with germination besides nutritional role. In chickpea, different secretory protein database (secretome) has developed from suspension culture very recently (Gupta *et al.*, 2011). Unfortunately peptide secretome database has not yet available, through which functional characterization of isolated peptides may be predicted.

In conclusion, isolated oligopeptides from one week old chickpea may play various physiological roles associated with germination and growth of seedlings at their early stages. Suitable application of these peptides or priming may be beneficial for improved germination but needs further in-depth investigations for establishing the exact molecular mechanism. For appropriate identification and determining structure-function relationship of these oligopeptides, more sophisticated purification module should be followed after large scale isolation of peptides from chickpea.

REFERENCES

Afzal I, Ashraf S, Qasim M, Basra SMA, Shahid M. 2009. Does halopriming improve germination and seedling vigour in marigold (*Tagetes* spp.) *Seed Sci. Technol.* 37:436-445

Agbola FW, Bent MJM, Rao PP, Kelley TG. 2000. Factors influencing the demand for chickpea in India: Implications for marketing and promotion in the Indian chickpea market. Proceedings of the 43rd Conference of Australian Agricultural and Resource Economics Society, Sydney, Australia, pp. 23-25.

Ali S, Bano A. 2008. Leaf and nodule senescence in chickeapea (*Cicer arietinum* L.) and the role of plant growth regularors. *Pak. J. Bot.* 40(6):2481-2492

Anil GK, Koundal R, Sik S. 1985. Senescence of attached leaves: Regulation by developing pods. *Physiol. Plant.* 63:87-92

Arnon DL.1949. A copper enzyme is isolated chloroplast polyphenol oxidase in *Beta vulgaries*. *Plant Physiol.* 24:1-15

Basra SMA, Zia MN, Mehmood T, Afzal I, Khaliq A., 2003. Comparision of different invigoration techniques in wheat (*Triticum aestivum* L.) seeds. *Pak J. Arid Agric.* 5:6-11

Bhatt ID, Rawal RS, Dhar U. 2000. Improvement in seed germination of *Myrica esculenta* Buch.- Ham. ex D. Don - a high value tree species of Kumaun Himalaya, India. *Seed Sci.Tech.* 28:597-605

Blom-Zandstra M, Pot CS, Maas FM, Schapendonk HCM. 1995. Effects of different light treatments on the nocturnal transpiration and dynamics of stomatal closure of two rose cultivars. *Sci. Hortic.* 61:251-262

Casenave EC, Toselli ME. 2007. Hydropriming as a pre-treatment for cotton germination under thermal and water stress conditions. *Seed Sci. Technol.* 35:88-98

Chaloupkova K, Smart CC. 1994. The abscisic acid induction of a novel peroxidase is antagonized by cytokinins in *Spirodela polyrrhizal* L. *J Plant Physiol.* 105:497-507

Das VSR, Rao IM, Raghavendra AS. 1976. Reversal of abscisic acid induced stomatal closure by benzyl adenine. *New Phytol.* 76:449-452

Erdal S, Genisel M, Turk H, Gorcek Z. 2012. Effects of progesterone application on antioxidant enzyme activities and K^+/Na^+ ratio in bean seeds exposed to salt stress Toxicol Ind Health. 28(10):942-946

Fincher GB. 1989. Molecular and cell biology associated with endosperm mobilization in germinating cereal grains. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 40:305-346

Gaur PM, Krishnamurthy L, Kashiwagi J. 2008. Improving drought-avoidance root traits in chickpea (*Cicer arietinum* L.): Current status of research at ICRISAT. *Plant Prod. Sci.* 11: 3-11

Ghosh A, Mandal P, Sircar PK. 2010. Wheat (*Triticum aestivum*) peptide(s) mimic gibberelic action and regulate stomatal opening. *Indian J. Exp. Bot.* 48:77-82

Gupta Ak, Singh J, Kaur N, Singh R. 1993. Effect of polyethylene glycol-induced water stress on uptake, interconversion and transport of sugars in chickpea seedlings. *Plant Physiol. Biochem.* 31:743-747

Gupta SK, Singhvi IJ, Shirsat M, Karwani G, Agarwal A, Agarwal A. 2011. Buccal adhesive drug delivery system: A review. *Asian J. Biochem. Pharm. Res.* 1:105-114

Habib N, Ashraf M, Ahmad MSA. 2010. Enhancement in seed germinability of rice (*Oryza sativa* L.) by pre-sowing seed treatment with nitric oxide (NO) under salt stress. *Pak. J. Bot.* 42:4071-4078

Hajouj T, Michelis R, Gepstein S. 2000. Cloning and characterization of receptor like protein kinase gene associated with senescence. *Plant Physiol.* 124:1305-1314

Hay RKM, Pedersen K. 1986. Influence of long photoperiod on the growth of timothy (*Phleum pratense* L.) varieties from different latitudes in northern Europe. *Grass Forage Sci.* 41:311-317

Islam MT. 2008. Dynamic rearrangement of F-actin organization triggered by host-specific plant signal is linked to morphogenesis of *Aphanomyces cochlioides* zoospores. *Cell Motil Cytoskel.* 65(7):553-562

Jeong YO, Cho JL, Kang SM. 1994. Priming eff ect of pepper (*Capsicum annum* L.) as aff ected by aging and growth regulators treatments. *J. Kor. Soc. Hort. Sci.* 35:407-414

Joyce SS, Thomas RS. 1980. Leaf senescence and abscisic acid in leaves of field-grown soybean. *Plant Physiol.* 66:1164-1168

Jukanti AK, Gaur PM, Gowda CLL and Chibbar RN. 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *Br. J Nutr.* 108:11-26

Kaur S, Brar KS, Sekhon BS, Joshi N, Shenhmar M, Singh J. 2000. Role played by Campoletis chlorideae Uchida in natural mortality of *Helicoverpa armigera* (Hübner) on chickpea in Punjab. *J. Biol. Control.* 14(1):51-54

Kaur S, Gupta Ak, Kaur N. 1998. Gibberellic acid and kinetin partially reverse the effect of water stress on germination and seedling growth in chickpea. *Plant Growth Reg.* 25:29-33

Kaur S, Gupta AK, Kaur N. 2000. Effect of GA3, kinetin and indole acetic acid on carbohydrate metabolism in chickpea seedlings germinating under water stress. *Plant Gr. Reg.* 30:61-70

Kaya MD, Okçu G, Atak M, Çıkılı Y, Kolsarıcı O. 2006. Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.). *Eur. J. Agron.* 24:291-295

Kelley TG. 1999. The fundamentals of the Indian sub-continent pulse economy: Long-*term implications of the Australian pulse industry*. GRDC Project No. VF35 A.

Khan AA. 1992. Preplant physiological seed conditioning. *Horticul. Rev.* 13:131-181

Khan AL, Shinwari ZK, Kim YH, Waqas M, Hamayun M, Kamran M, IJ Lee. 2012. Role of endophyte *chaetomium globosum* lk4 in growth of *capsicum annuum by* producion of gibberellins and indole acetic acid *pak. J. Bot.* 44(5):1601-1607

Khan AL, Hamayun M, Kim YH, Kang SM, Lee JH, Lee IJ. 2011. Gibberellins producing endophytic *Aspergillus fumigatus* sp. LH02 influenced endogenous phytohormonal levels, plant growth and isoflavone biosynthesis in soybean under salt stress. *Process Biochem.* 46:440-447

Kirk JTO. 1968. Studies on the dependence of chlorophyll synthesis on protein synthesis in *Euglena gracilis* together with a nomognam for determination of chlorophyll concentration. *Planta.* 78:200-207

Kirk JTO, Allen RL. 1965. Dependence of chloroplast pigment synthesis on protein synthesis: Effect of actidione. *Biochem. Biophys. Res. Commun.* 21:523-530

Knapp AK, Smith WK. 1989. Influence of growth form on eco-physiological responses to variable sunlight in subalpine plants. *Ecology.* 70:1069-1082

Ladizinsky G, Adler A. 1976. The origin of chickpea, *Cicer arietinum* L. *Euphytica.* 25:211-217

Ma ZL, Wang YP, Wang CX, Miao FZ, Ma WX. 1997. Reversed-phase ion-pair high performance liquid chromatographic determination of Co(II)-, Ni(II)-,V(V)-and Fe(III)-2-(2-benzothiazolylazo)-5-(3-sulforpropy)aminophenol chelates. *Talanta.* 44:743-748

Monerri C, Garcia LA, Guardiola JL. 1986. Sugar and starch changes in pea cotyledons during germination. *Physiol. Plant.* 67:49-54

Murtaza G, Asghar R. 2012. α-Amylase activities during seed development and germination in pea (*Pisum sativum* L.) treated with salicylic acid. *Pak. J. Bot.* 44(6):1823-29

Nawaz K, Ashraf M. 2010. Exogenous application of glycine betaine modulates activities of antioxidants in maize plants subjected to salt stress. *J. Agron. Crop Sci.* 196:28-37

Negi A, Boora P, Khetarpaul N. 2001. Starch and protein digestibility of newly released moth bean cultivars: Effect of soaking, dehulling, germination and pressure cooking. *Nahrung.* 45(4):251-254

Oelkers K, Goffard N, Weiller GF, Gresshoff PM, Mathesius U, Frickey T. 2008. Bioinformatic analysis of the CLE signaling peptide family. *BMC Plant Biol.* 8:1

Pedroche J, Yust MM, Giron-Calle J, Alaiz M, Millan F, Vioque J. 2002. Utilisation of chickpea protein isolates for production of peptides with angiotensin I-converting enzyme (ACE)-inhibitory activity. *J. Sci.Food Agric.* 82:960-965

Ramakrishna BA, Sharma HC, Subbaratnam GV, Sharma KK. 2005. Development of transgenic chickpea (*Cicer arietinum* L.) with Bt cry1Ac gene for resistance to pod borer, Helicoverpa armigera. In: Food legumes for nutritional security and sustainable agriculture, Indian Agricultural Research Institute (IARI), New Delhi, India, pp. 58.

Samet JS, Sinclair TR. 1980. Leaf senescence and abscisic acid in leaves of field-grown soybean. *Plant Physiol.* 66(6):1164-1168

Saraf CS, Rupela OP, Hegde DM, Yadav RL, Shivkumar BG, Bhattarai S, Razzaque MA, Sattar MA. 1998. *Biological Nitrogen Fixation and Residual Effects of Winter Grain Legumes in Rice and Wheat Cropping Systems of the Indo-Gangetic Plain.* In: Residual Effects of Legumes in Rice and wheat Cropping Systems of the Indo-Gangetic Plain. Rao K, Johansen JVDK, Rego CTJ. (eds.), Oxford and IBH Publishing Co. Pvt. Ltd, New Delhi, India, pp.14-30.

Shakya MS, Patel MM, Singh VB. 2008. Knowledge level of chickpea growers about chickpea production technology. *Indian Res. J. Ext. Edu.* 8:65-68

Siveritepe HO, Dourado AM. 1995. The effect of priming treatment on the viability and accumulation of chromosomal damage in aged pea seeds. *Ann. Bot.* 75:165-171

Srinivasan K, Saxena S, Singh BB. 1999. Osmo- and hydropriming of mustard seeds to improve vigour and some biochemical activities. *Seed Sci. Technol.* 27:785-793

Talha M, Larsen P. 1975. Effect of abscisic acid on the transpiration of *Zea mays*. *Physiol Planta.* 33:66

Thakare KG, Chore CN, Deotale RD, Kambale PS, Lende SR. 2006. Influence of nutrients and hormones on biochemical and yield and yield contributing parameters of soybean. *J. Soils Crops.* 16(1):210-216

Upadhyaya HD, Kumar S, Gowda CLL, Singh S. 2006. Two major genes for seed size in chickpea (*Cicer arietinum* L.). *Euphytica.* 147:311-315

Villalobos N, Martin L. 1992. Involvement of cytokinins in the germination of chick-pea seeds. *Plant Growth Regul.* 11:277-291

Wahid A, Sehar S, Perveen M, Gelani S, Basra SMA, Farooq M. 2008. Seed pre-treatment with hydrogen peroxide improves heat tolerance in maize at germination and seedling growth stages. *Seed Sci. Technol.* 36:633-645

West CE, Waterworth WM, Stephens SM, Smith CP, Bray CM. 1998. Cloning and functional characterization of a peptide transporter expressed in the scutellum of barley grain during the early stages of germination. *Plant J.* 15:221-230

Ye XE, Ng TB. Rao PF. 2002. Cicerin and arietin, novel chickpea peptides, with different antifungal potencies. *Peptides.* 23:817-822

Ye XY, Ng TB, Tsang PWK, Wang J. 2001. Isolation of a homodimeric lectin with antifungal and antiviral activiral activities from red kidney bean (*Phaseolus vulgaris*) activities. *J. Protein Chem.* 20(5):367-375

Zhang L, Ma XL, Zhang Q, Ma CL, Wang PP, Sun YF, Zhao YX, Zhang H. 2001. Expressed sequence tags from a NaCl-treated Suaeda salsa cDNA library. *Gene.* 267:193-200