Allelopathic Effect of Chrysanthemum Procumentens on the Seed Germination and Seedling Growth of Beans Phaseolus Vulgaris

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Abstract

The present study was undertaken to assess the allelopathic effect of *Chrysanthemum procumentens* in relation to germination and growth of bean seedling. *C. procumentens* reduced germination and suppressed early seedling growth of beans. With increase in extract concentration from 20 mg ml^{-1} to 60 mg ml^{-1}, a gradual decrease in seed germination evaluated by reduction in the concentration of biomolecules such as total protein, total soluble sugar and amylase activities; and increase in the activities of hydrolytic enzymes such as proteases, Lipases and Arginase occurred. The longest seedling root and shoot lengths were recorded on day 1 at 20 mg ml^{-1} extract concentration while the shortest seedling root and shoot length were recorded on day 6 at 80 mg ml^{-1} extract concentrations. It was noted that the reduction in germination and suppression of seedling growth observed in this study were concentration and time dependent. The result suggested that *C. procumentens* had good allelopathic potential which reduces germination and plant growth.

Keywords Allelopathy, Chrysanthemum Procumentens, Seedling Growth, Germination.

1.   Introduction

The word "allelopathy" comes from the Greek words "allelon" meaning mutual and "pathos" meaning harm respectively. Austrian botanist, Hans Molish describe allelopathy as the result of biochemical interactions between plants (Putnam and Duke, 1978). It can also be defined as the direct or indirect biotic interaction of a plant on another plant by the release of chemicals called allelochemicals from different parts of the plant into the environment (Rice, 1984). Allelochemicals are products of secondary metabolism and are non-nutritional primary metabolites (Iqbal and Fry, 2012).

They vary in chemical composition, concentration and localization in plant tissue (Inderjit and Duke, 2003) and may be present in all plant organs including leaves, flowers, fruits, roots, rhizomes, stems and seeds (Putnam and Tang, 1986). Allelochemicals exert different effects on the synthesis, functions, contents and activities of various enzymes. Exposure of plants to allelochemicals affect their growth and development including germination and early seedling growth (Macias et al., 2007).

These morphological changes can be caused by a variety of more specific effects acting at the cellular or molecular level in the receiver plants (Zhang et al., 2010). Allelopathic effect is mainly referred to as a type of negative interaction (De-Alluquerque et al., 2011) but positive interactions have also been reported depending on the allelochemicals considered, the target plant and the concentration tested (Eichenberg et al., 2014). *C. procumentens* is a small creeping herb that was first cultivated in China in the 15th Century and by 1630, over

500 cultivars were already on record. *C. procumentens* is native to Asia and North East Europe but it is not indigenous to Nigeria.

In culinary industry, the yellow or white flowers are used to make sweet drinks in Asia (Gu et al., 2008) while the leaves are used in China to prepare thick snake soup. It was also used in Agriculture as natural source of insecticide. "Asteraceae" consumed in the Western and Eastern part of Asia and China has been reported to possess allelopathic potentials (Cheng 2012; Prakash et al., 2012). The observation that other plants species seized to grow when *C. procumentens* was domesticated close to them in Nigeria and that no work has been reported on *C. procumentens* domesticated in Nigeria led to the need to investigate its allelopathic potential. This study is therefore designed to unveil some of the primary effect of *C. procumentens* on biochemical parameters which underlie its visible physiological effect.

2. Materials and methods

2.1 Plant Extract.

Fresh leaves of *C. procumetens* were collected from Senior Staff Quarters, Obafemi Awolowo University, Ile-Ife in Osun State, Nigeria and was identified and authenticated at the Department of Pharmacy Herbarium, Obafemi Awolowo University, Ile-Ife. The leaves of *C. procumetens* were air-dried at room temperature for 4 weeks. They were pulverized and 214 g of the powdered leaf sample was extracted in 1.2 litres of 70% methanol for 72 hours. The mixture was filtered through filter paper (Whatman number 1) and the filtrate concentrated to dryness using a rotary evaporator.

2.2. Experimental Treatments

Three different concentrations of the methanolic extract of *C. procumetens,* that is 20, 40 and 60 mg ml^{-1} were taken for the seed germination inhibition tests while four different concentrations of *C. procumetens* (20, 40, 50 and 80 mgml^{-1}) were taken for the seedling growth inhibition tests.

2.3 Seed Germination Inhibition Test

The germination of bean seeds was studied by Petri dish method. Seven (7) bean seeds each was placed in twelve (12) Petri dishes lined with double layer of filter paper. The Petri dishes were grouped into four, groups of three Petri dishes. Each of the group was treated with five (5 ml) of the methanolic extracts of *C. procumetens,* in the three concentrations (20, 40 and 60 mg/ml) daily while the remaining group served as the control which received distilled water. The Petri dishes were kept at room temperature (28 ± 1^{0}C.). One (1) seed was harvested daily from each petri dish (triplicates), homogenized in phosphate buffer (5 ml) and kept in refrigerator until required. The following assays were carried out on the homogenized bean seeds

2.3.1 Estimation of the Total Soluble Sugar

The amount of total soluble sugar in the homogenized seed was estimated by the phenol sulphuric acid method (Dubois et al., 1956). Five percent phenol solution (0.2 ml) was added to 40 µl of the homogenized bean seeds followed by the addition of 1 ml of concentrated sulphuric acid. The mixture was vortexed and incubated in a water bath at 25-30 ^{o}C for 20 minutes and allowed to cool. Absorbance was then read at 490 nm. Dilutions of glucose standards were made and used for the calibration of standard curve.

2.3.2 Estimation of protein concentration

The protein concentration of the bean seed homogenates was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard protein. The protein content was expressed as mg ml^{-1} of extract. The bean seed homogenate (0.2 ml) was added to 0.8 ml of phosphate buffer followed by the addition of 0.4 ml of Bradford reagent and the absorbance was then read at 595 nm.

2.3.3. In vitro alpha amylase activity of bean seeds

Boiled and fresh bean seeds homogenate (0.02 and 0.05 ml respectively) were incubated with 0.2 ml of 1% soluble starch and the volume was then made up to 2 ml with phosphate buffer (pH 6). The mixture was incubated at room temperature for 5 min followed by the addition of 0.5 ml of DNSA. The reaction mixture was boiled for 5 min and absorbance was read at 540 nm. Sugar released was calculated taking into cognizance the amount of residual sugar that was originally present in the homogenate, which was then subtracted from the total sugar estimated from the calibration curve. A unit of amylase activity was defined as the amount of enzyme that catalysed the liberation of reducing ends from bean seeds starch equivalent to 1 µg of D-glucose per minute (Adewale et al., 2012). Enzyme activity was calculated using this expression:

$$\text{Activity} = \frac{OD}{\text{slope of standard curve} \times \text{vol. of enzyme} \times \text{incubation time}}$$

2.3.4. In vitro lipase activity of bean seeds

Lipase activity in bean seeds homogenate was assayed spectrophotometrically using p-nitrophenyl laurate (p-NPL) method as described by Vorderwulbecke et al., (1992) with slight modifications. The emulsion was prepared by first dissolving 0.001 g of p-NPL in 1 ml of isopropanol and then mixed with 9 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 50 µl triton X-100 and 0.01 g gum Arabic. About 700 µl of the mixture was added to 300 µl of the appropriately diluted enzyme solution. The liberated p-nitrophenol was monitored by the change in absorbance at 410 nm at an interval of 15 secs over a 3 min period. One unit (1U) of enzyme activity is defined as the amount of enzyme that released 1 µmol of p-nitrophenol from p-NPL in one minute (ε = 15600 $M^{-1}cm^{-1}$) under the assay conditions. Enzyme activity was calculated using the formula:

Lipase activity = $\underline{\Delta \text{Abs}_{410 \text{ nm/min}} \text{ X V}}$

ε X v

Δ Abs $_{410 \text{ nm/min}}$ = change in absorbance at 410 nm

V   = total volume of the assay mixture

v   = volume of enzyme solution used for the assay

ε = extinction coefficient = 15,600 $M^{-1}cm^{-1}$

2.3.5. In vitro protease activity of bean seeds

The activity of protease enzyme in bean seeds was measured in terms of its action on protein, and this was determined spectrophotometrically. The bean seeds were homogenized in 1.9 ml of 0.1M tris-HCl buffer (pH 9.0) and 0.1 ml of the homogenate was added to a test tube containing 1.0 ml of 0.5% casein.

The reaction mixture was then incubated at 37°C for 15, 30, 45 and 60 minutes in a water bath. The reaction was arrested by the addition of 2.0 ml of 5% trichloroacetic acid. The mixtures were then centrifuged and the supernatants collected. This was followed by the addition of 1.0 ml of Folin-Ciocalteu to 0.5 ml of the supernatants. The resultant solution was allowed to stand for 30 min for colour development.

The absorbance of the tube was read at 280 nm and the optical density obtained was extrapolated from the tyrosine standard curve to determine the concentration of the tyrosine liberated by the protease enzyme produced. The activity of the enzyme was calculated for the different concentrations of tyrosine determined and recorded (Chidi et al., 2015).

2.3.6 In vitro Arginase Activity of Bean Seeds

Arginase activity in bean seeds homogenate was measured by the rate of urea formation from the bean seed homogenate evident by its colour reaction with Ehrlich's reagent a modification of the methods of Hagam and Dallam (1968) and Kaysen and Strecker (1973). Typically, 100 µl of an appropriate diluted bean seed homogenate was added to a solution containing 100 µl of 0.33M arginine and 150 µl of 2.0 mM tris-HCl buffer (containing 50 µl of 1 mM $MnCl_2$, pH 9.5) of 400 µl in final volume.

It was then incubated for 10 min at 37°C, after which 1.25ml of Erhlich reagent was added and the absorbance was read spectrophotometrically at 450 nm. The standard curve was prepared using varying concentrations of urea made to equal volume of 400 µl with distilled water. The arginase activity was calculated from the standard curve (Kaysen and Strecker, 1973).

2.4 Seedling growth inhibition test

Beans seeds (5 each) were planted in 15 petri dishes. After two weeks of planting, the petri dishes were divided into 5 groups (3 petri dishes per group). Groups 1, 2, 3 and 4 were treated with 2 ml of 20, 40, 50, 80 mg/ml of the *C. procumetens* methanolic extract respectively for six days, while group 5 which received distilled water served as the control group. The shoot and root lengths were measured daily, harvested, homogenized and kept in the refrigerator for further use.

2.4.1 Estimation of Total Soluble Sugar Concentration in Root of Growing Bean Plant

Total soluble sugar was estimated in the root of the growing plant using anthrone-sulphuric acid reaction method as described by Farhad et al., (2011). Bean roots (1 g) was homogenized in 5 ml of 95% ethanol. The homogenates were centrifuged at 4000 rpm for 10 min and the supernatant collected. To 1.0 ml of the supernatant, 3.0 ml of freshly prepared anthrone reagent (150 mg anthrone in 100 ml of 72% sulphuric acid) was added. The mixture was incubated in boiling water for 10 minutes and then cooled. The absorbance was read at 625 nm against reagent blank. The sugar content was calculated from the glucose standard curve (Anjorin et al., 2016).

2.4.2 Estimation of total protein content in root of growing bean plant

The protein concentration was estimated according to Bradford, (1976). Beans roots (1 g) were homogenized in 5 ml of distilled water. The homogenates were centrifuged at 4000 rpm for 10 minutes. The homogenate (0.2 ml) was made up to 1 ml using distilled water and then added to the test tubes containing 0.4 ml of Bradford reagent. The absorbance was read at 595 nm. The protein content was calculated from the protein standard curve using BSA (bovine serum albumin).

2.5 Data Analysis

The data were subjected to one-way analysis of variance, and treatment means were compared p<0.05 by Ducan multiple range test. Statistical analysis was done with SPSS 18 for windows statistical software package (SPSS, Chicago, IL, USA).

3. Results

3.1 Sugar content in bean seeds treated with *C. procumetens* leaf extract

There was a reduction in sugar content of bean seeds with increase in extract concentration and germination period (Table 1). The highest sugar content was observed at day 1 (20 mg ml^{-1}) while the least sugar content was recorded at day 6 (60 mg ml^{-1}). Table 1: Sugar content in bean seeds treated with *C. procumetens* Leaf Extracts (Seed Germination)

Conc.	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
20	88.9±1.6	84.2±2.9	82.3±2.5	79.7±0.8	76.6±0.3	57.2±0.0
40	88.5±0.9	83.9±2.5	82.2±1.9	79.4±0.7	72.3±0.5	47.3±0.6
60	87.3±0.6	82.5±4.1	81.2±1.3	79.3±1.5	65.5±0.5	29.9±1.3
CTRL	89.5 ±0.4	85.7 ±6.2	81.0 ±1.1	80.6± 0.9	79.4 ±0.2	62.0 ±0.4

Data are expressed as Mean ± SEM, (n = 3). Conc. = Concentration expressed in mg ml^{-1}

3.2 Protein content in bean seeds treated with *C. procumetens* leaf extract

Protein content decreased with increase in concentration of the extract as well as with increase in germination period (Table 2). The highest protein content was observed at day 1(20 mg ml^{-1}) while the least protein content was observed at day 6 (60 mg ml^{-1}).

Table 2: Protein content in bean seeds treated with *C. procumetens* leaf extract

Conc. mg/ml	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
20	369.0±16.24	259.1±1.6	250.0±1.1	230.4±4.8	222.9±0.2	185±1.00
40	297.9±12.5	257.5±1.5	245.8±1.5	227.9±7.9	217.5±0.4	128.7±2.1
60	263.7±6.1	255.4±0.1	240.8±4.2	226.6±14.0	202.5±1.6	125.0±1.4
CTRL	382.0 ±3.0	259.0± 6.5	254.0 ±2.4	234.0 ±4.3	200.0± 6.0	190.0 ±1.2

Data are expressed as Mean ± SEM, (n = 3). Conc. = Concentration expressed in mg ml^{-1}

3.3 Alpha-amylase activity in bean seeds treated with *C. procumetens* leaf extract

Reduction in alpha-amylase activity was observed in a dose dependent manner as well as with increase in germination period in bean seeds treated with *C. procumetens* leaf extract (Figure 1A). The highest alpha amylase activity was recorded on day 1 (20 mg ml^{-1}) while the least activity was recorded on day 6 (60 mg ml^{-1})

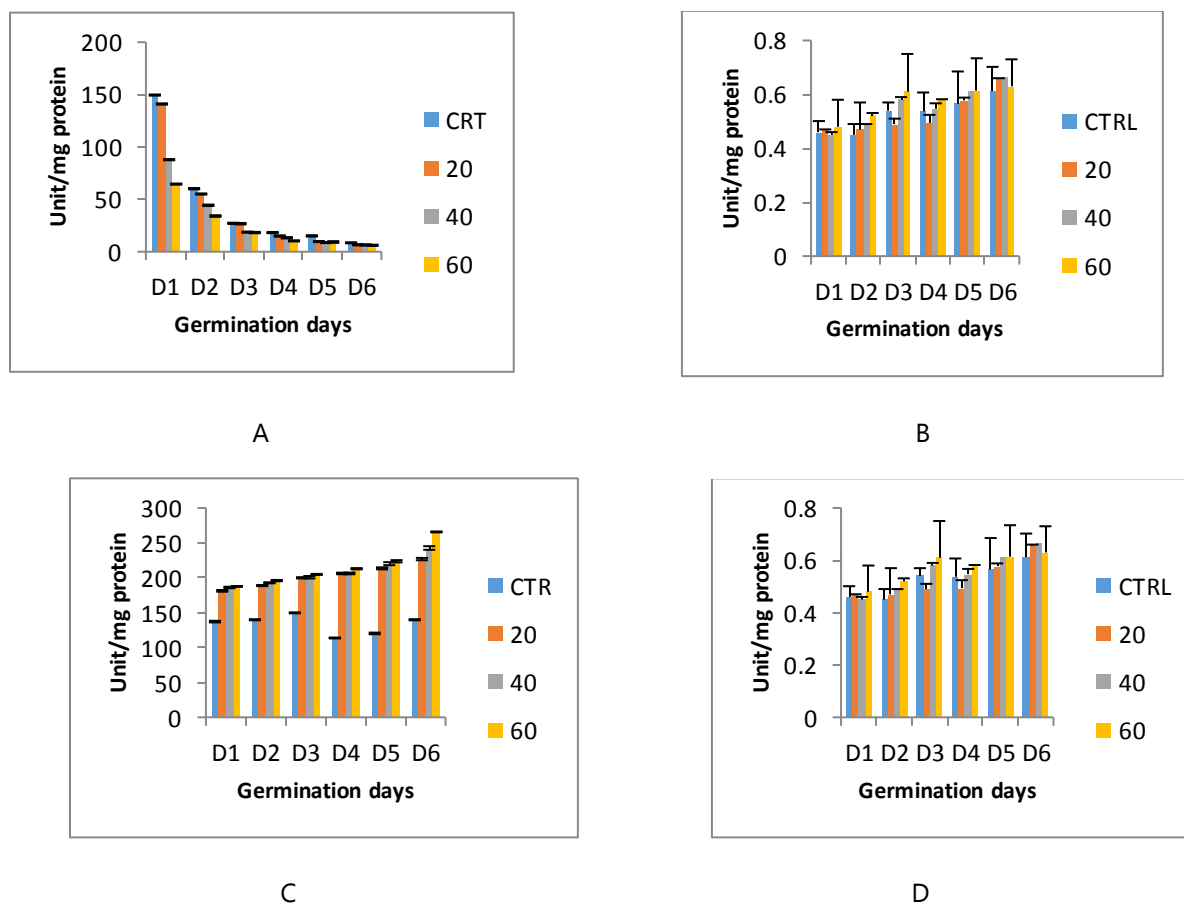## 3.4 Protease activity in bean seeds treated with *C. procumetens* leaf extract

Protease activity increased with increase in concentration of the extract as well as with increase in germination period (Figure 1B). The highest protease activity was recorded on day 6 (60 mg ml^{-1}) while the least activity was recorded on day 1 (20 mg ml^{-1}).

3.5 Lipase activity in bean seeds treated with *C. procumetens* leaf extract

Lipase activity increased with increase in concentration of the extract as well as with increase in germination period (Figure 1C). The highest lipase activity was recorded on day 6 (60 mg ml^{-1}) while the least activity was recorded on day 1 (20 mg ml^{-1}).

3.6 Arginase activity in bean seeds treated with *C. procumetens* leaf extract

The arginase activity increased with increase in concentration of the extract as well as with increase in germination period (Figure 1D). The highest arginase activity was recorded on day 6 (60 mg ml^{-1}) while the least activity was recorded on day 1 (20 mg ml^{-1}).

A

B

C

D

Figure 1: Enzyme activity of *C. procumentens* treated bean seeds per germination days

A – alpha-amylase activity; B – protease activity; C – lipase activity; D – arginase activity

3.7 Effect of *C. procumentens* leaf extract on beans seedling root length

With increase in extract concentration, there was a gradual decrease in seedling root length as the days of treatment increased (Table 3). The longest root length was recorded on day 1(20 mg ml^{-1}) while the shortest root length was recorded on day 6 (80 mg ml^{-1}).Table 3 Effect of *C. procumentens* Leaf Extract on Beans Seedling Root Length in cm

Days	Water	20 mg/ml	40 mg/ml	50 mg/ml	80 mg/ml
1	10.5±0.00[a]	8.7±0.03[a]	5.5±0.02[a]	5.0±0.01	5.0±0.03[c]
2	11.0±0.00	8.4±0.03	5.1±0.01	4.8±0.00[b]	4.8±0.02
3	11.2±0.01[a]	8.0±0.00[a]	5.0±0.02[b]	4.4±0.02	4.7±0.00[a]
4	11.6±0.00	7.8±0.02	4.8±0.02[b]	4.20±0.01[a]	4.7±0.01
5	12.0±0.00[b]	7.0±0.03[b]	4.5±0.01	4.1±0.00	4.5±0.00[c]
6	12.2±0.00	7.0±0.00	4.3±0.00	4.0±0.00[c]	4.3±0.01

Data are expressed as mean ± SEM, (n=3). Values with [a] superscript are statistically different (p<0.05)

3.8 Effect of *C. procumentens* leaf extract on beans seedling shoot length

The bean seedling shoot length was observed to decrease as the concentration of *C. procumentens* leaf extract increased and as the days of treatment increased (Table 4). The longest shoot length was recorded on day 1 (20 mg ml^{-1}) while the shortest shoot length was recorded on day 6 (80 mg ml^{-1}).

Table 4: Effect of the *C. procumentens* leaf extract on bean seedling shoot length in cm

Days	Water	20 mg/ml	40 mg/ml	50 mg/ml	80 mg/ml
1	26.0±0.00	25.0±0.00[a]	23.0±0.00	20.0±0.03[b]	19.0±0.01[a]
2	24.0±0.01[a]	21.0±0.01	23.0±0.00[c]	19.0±0.00[c]	17.0±0.02
3	22.0±0.00	20.0±0.00[a]	22.0±0.01[a]	18.0±0.02	14.0±0.03[b]
4	20.0±0.00[b]	21.0±0.03	20.0±0.00[b]	17.5±0.01[a]	12.0±0.00
5	18.0±0.00[a]	19.0±0.02[b]	18.0±0.02	17.0±0.00[b]	10.0±0.01[c]
6	17.2±0.01	16.0±0.01[a]	17.5±0.00	16.8±0.01	9.8±0.02[b]

Data are expressed as mean ± SEM, (n=3). Values with [a] superscript are statistically different (p<0.05)

3.9 Total Sugar and Protein Contents in the Root of Growing Beans Plant

The Sugar and protein content reduced in treated bean seedling with increase in concentration of the extract (Table 5).

Table 5: Total Sugar and Protein Contents in Treated Bean Seedlings

Conc. (mg/ml)	Sugar content (mg/ml)	Protein content (mg/ml)
20	0.43 ± 0.03	4.57 ± 0.65[c]
40	0.14 ± 0.04[c]	3.57 ± 0.33[b]
50	0.10 ± 0.00[a]	2.57 ± 0.71[a]
80	0.06 ± 0.07[a]	1.18 ± 0.58[a]
Negative control	0.61 ± 0.02[b]	6.23 ± 0.02[a]

Data are expressed as Mean ± SEM, (n = 3).

4. Discussion

Since its discovery in 1930s so many investigations have been carried out to elucidate the biochemical mechanisms underlying allelopathy. The visible allelopathic effects observed as inhibited or delayed seed germination or reduced seedling growth are known to be the secondary expressions of primary effects on

biochemical processes such as cell division, cell differentiation, ion and water uptake, water status, phytochrome metabolism, respiration, photosynthesis, enzyme function, signal transduction as well as gene expression (Inderjit and Duke, 2003; Macias et al., 2007). It is however pertinent to have an understanding of biochemical mechanisms responsible for the observed allelopathic effect of *C. procumentens*.

Results obtained from the investigation of the allelopathic effect of *C. procumetens* on germinating bean seeds showed that the concentration of sugar and protein in the treated seeds reduced in a dose dependent manner. This implies that the storage proteins and total sugar are degraded and used for seed germination (Bewley and Black, 1994). Correlation between seed reserve content and germination rate has been reported (Soriano et al., 2014). Soluble sugar (such as glucose and sucrose) was positively correlated with germination percentage of *Medicago truncatula* seeds (Vandecasteele et al., 2011).

Protein content was positively correlated with germination percentage of *Pinus pinaster* (Wahid and Bounoua, 2013). Alpha amylase hydrolysis the alpha glycosidic linkages in polysaccharide chains to form simple sugar that is needed to generate enough energy for plant survival and growth. It was observed that treatment with *C. procumentens* caused a reduction in alpha-amylase activity in treated bean seeds in a dose dependent manner and with increasing time of germination. The treatment caused de-activation of amylase activity which was well correlated with decrease in sugar content in the endosperms under similar conditions. Inhibition of alpha amylase activity as pre-biochemical changes in plants under stress have been reported (Ramakrishna and Ramakrishna, 2005).

In this study, beans treated with *C. procumentens* showed significant increase in protease activity during germination. Proteases which breaks down peptide bonds in protein molecules into smaller peptide chains was activated during seed germination due to the removal of inhibition factor by the seed germination process and thus allowing the activation of stored proteases (Muntz, 1996). This led to increased concentration of soluble amino acids and decreased amount of storage protein. Extract treated been seed showed significant increase in lipase activity as concentration and days of germination increased.

This observation is in agreement with the report that during germination lipid reserve is rapidly used up in the production of energy for embryonic growth due to very high lipolytic activity during this period resulting in increased concentration of fatty acids and decreased amount of lipid reserve (Dkhil and Denden, 2010). Arginase a key enzyme involved in the balance of soil nitrogen needed for the survival of germinating seed was found to increase in a concentration dependent manner as the time of germination increased in this study. This result is in agreement with Ona et al., (2005) who reported increase in arginase activity during stress condition.

The longest mean seedling root and shoot length were observed in 20 mg ml^{-1} concentration while the shortest seedling mean root and shoot length were found in 80 mg ml^{-1} concentration. The result indicated that the effects of *C. procumentens* on the seedling root and shoot lengths were concentration dependent which indicated that lower concentrations can stimulate plant growth while higher concentration cause growth inhibition. This can be attributed to the fact that low dose of phenolic compounds stimulates protein synthesis and activation of antioxidant enzymes (Baziramakenga et al., 1995) which are effective in plant protection (Kleiner et al., 1999), while high levels of phenolic application result in plant damage (Politycka et al., 2004). This result is in agreement with findings of earlier studies (Swain et al., 2008; Ghareib et al., 2010).

The total protein and soluble sugar content of the growing bean seedling decreases significantly as the concentration and days of sowing increased. The homogenized bean seedling at 20 mg ml^{-1} had the highest total protein and sugar content while the seedling homogenate at 80 mg ml^{-1} had the least protein and sugar content. The total protein content decreased possibly due to the decrease rate of protein synthesis and increased rate of proteolysis (Yuan et al., 1998). It can be said that reduction in seedling vigor and seed germination in bean seeds under stress situations may possibly be due to allelochemicals released which alters the activities of hydrolytic enzymes such as amylase, lipase, protease and arginase leading to changed levels of substrate like starch, sugars, fatty acids, lipids protein, amino acids.

5. Conclusion

The present study revealed that the extract of *C. procumentens* was highly effective against seed germination and seedling growth of beans. Possibly through the released allelochemicals which resulted in changes in gene expression or alteration of enzyme activity. This may play important role in weed control and can also be used as alternative to chemical compounds. Furthermore, these allelochemicals responsible for germination and growth reduction could be isolated and identified.

Contribution

k. F. Akinwunmi designed the work, E.A. Odunsi performed the experiment, C. V. Amadi contributed to data analysis, K.F. Akinwunmi and C. V. Amadi wrote the manuscript.

Acknowledgements

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