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Gene expression of 49 kDa apyrase, cytoskeletal proteins, ATPase, ADPase and amino acid contents of *Pisum sativum* (L.) cells germinated in *Euryops arabicus* (Steud. ex Jaub. & Spach) water extract

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Abstract: The present research reports of quick and marked changes induced by plant extract of *Euryops arabicus* in the gene expression of 49-kDa apyrases, cytoskeletal proteins, ATPases, ADPase and amount of amino acid of pea (*Pisum sativum* L. var. Alaska). Pellets of cytoskeletals proteins (27000 xg) were probed with anti-apyrase antibody, biotinylated anti-rat, actin and alpha and beta-tubulin for Western blotting. ATPase and ADPase activities were determined based on the hydrolytic efficacy of adenine triphosphate and adenine diphosphate. By 72 hours, the abundance of apyrases, cytoskeletal proteins and amount of amino acid in pellets of 27000 xg of germinated pea seeds in *E. arabicus* extracts were sharply increased than those sown in distilled water. All the samples exhibited that the stems had more amount from apyrases, cytoskeletal proteins, amino acids and ATPase and ADPase activities than primary leaves and primary roots that were germinated either on *E. arabicus* water extract or in distilled water. Based on the enzyme's capability to hydrolyse nucleotide triphosphate and nucleotide diphosphate as well as the direct association between expression of 49-kDa apyrase and cytoskeletal proteins, *E. arabicus* water extract had an important effect on plant germinations.

Introduction

The cytoskeleton of eukaryotic cells is a dynamic filamentous apparatus showing an interesting architecture (Li and Ren, 2015). The cytoskeletal proteins are mainly composed of a web of filaments that are distributed in the cell and in the nucleus as actin filaments and microtubules, respectively (Bershadsky and Vasilev, 1988). Threads of cytoskeletal proteins are associated with each another, or with other cytoskeletal proteins, or with various plant cell systems (Abe and Takeda, 1986, 1989).

It has been reported that cytoskeletal proteins play essential roles in most cellular activities in plants. For example, they direct the Golgi apparatus (Boevink *et al*., 1998), mitochondrial movements (Van Gestel *et al*., 2002), induce the myosin motor (DePina, AS, 1999), are involved in the energy supply for various metabolic activities (Mathur *et al*., 2002), are linked to Sertoli cell ectoplasmic specializations (Cavicchia *et al.,* 2011) and determine spindle position during cell division (Lloyd and Traas, 1988). Moreover, cytoskeletal elements organise the stimulation of gravitropism in root cell (Cyr, 1994), trichome branching and morphogenesis (Vantard *et al.,* 2001; Szymanski *et al*., 1999).

On the other hand, apyrase purified from pea plumule nuclei (NTPase, EC 3.6.1.15) is classified as a nucleoside triphosphatase through hydrolyses by both adenine triphosphate (ATP) and adenine diphosphate (ADP) (Moustafa *et al.,* 2003; Komoszynski and Wojtczak, 1996). Animal apyrases were found to be ectoapyrase and usually in combination with the extra-cellular matrix (ECM) as a membrane-bound protein tissue (Valenzuela *et al*., 1996; Battastini *et al*., 1998).

The real functions and locations of apyrases are not clearly apparent and many functions still ambiguous in nearly all plant cells – sometimes found as a soluble enzyme in the nuclei or cytoskeleton (Shibata *et al*., 1999). And as the apyrase compartments exhibited various locations, therefore assumed diverse functions, including phosphate uptake, toxin resistance, cytoskeleton-based cellular metabolism, had important functions during germination of pea seeds, phytochrome

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responses and involvement in potato starch metabolism (Handa and Guidotti, 1996; Shibata *et al*., 1999; Thomas *et al*., 1999, 2000; Moustafa *et al*., 2003 and others). Also, one report indicated that the apyrases present in the root were essential for nodulation and stimulating the *de novo* organogenesis of the emergent root nodule (Cohn *et al*., 1998).

Amino acids play several important roles in plants, from being the building blocks of all proteins to being involved in essential metabolites with several physiological activities, such as the transportation of ions and organic nitrogen, regulation of stomatal opening and heavy metal detoxification. They affect the activity and biosynthesis of some enzymes, redox-homeostasis and gene expression (Rai, 2002; Ortiz-Lopez *et al.,* 2000).

Also, the cycling of amino acids between roots and shoots, that are governed by the conducting tissues, xylem and phloem (Jeschke and Pate, 1991; Jeschke and Hartung, 2000), and the fluctuation in the concentrations that were either ascending or descending are thought to be controlled by various factors such as nitrogen, carbon availability and sulphur metabolism (Mariusz, 2013; Réjane and Guillaume, 2014). *E. arabicus*, as 7% crude water extract, caused marked stimulation in the shoot fresh weight, shoot length, root length and in fresh root as well as the dry weight of the seedling of *Vicia faba* L. (Moustafa *et al*., 2016).

To the best of our knowledge, there are no studies of the effect of specific plant extracts on amino acids biosynthesis in pea seedlings, linked with ATPase, 49-kDa apyrase and cytoskeletal proteins. In the present research, we used antibodies against both 49-kDa apyrase and the cytoskeletal proteins to measure changes in their amount. In addition, changes in the enzymatic properties of the ATPase and ADPase, in association with amino acids biosynthesis during the early stage of germination of Alaska peas grown in the crude water extracts of *E. arabicus*, have been estimated.

Materials and Methods

Sample preparation

Seeds of *Pisum sativum* L. var. Alaska were imbibed in distilled water (DW) for 10 hours and then allowed to germinate in DW by irrigation either by (DW) as a control or with a 7% water extract of *E. arabicus* for 72 hours at 21-23°C, (Moustafa *et al*., 2003, 2016). Seven grams dissected from stems, leaves and roots were separated from the cotyledons 72 hours after germination. Collected tissues were grounded on ice with a mortar and pestle in a cytoskeleton-stabilising buffer (CSB) of volume 7 consisting of 10 mM Mg $(OAc)_2$, 2 mM EGTA, 5 mM HEPES-KOH (pH 7.5), 1 mM PMSF and 0.5% PTE. The resulted homogenates were filtered using two layers of Mira cloth (Calbiochem) and centrifuged for 27,000 xg to collect cytoskeletal proteins.

Gel electrophoresis and Western blotting

According to the methods described by Moustafa *et al*., 2003, the sample buffer consisted of 2% 2-mercaptoethanol, 10 mM Tris-HC1, pH 6-8, and 2% LDS as a 2x stock for preparation. The buffer stock was mixed with an equal volume of 27,000 xg pellets, which were obtained, and boiled for three minutes; then the mixture was advanced for electrophoresis by SDS-PAGE. After electrophoresis, every gel was removed and adhered precisely, without any bubbles on a PVDF membrane (ImmobilonTM Transfer Membranes, Millipore), for protein transfers. After blotting, the PVDF membranes were transferred into sterilised sealed bags and probed with the antibodies, namely anti-apyrase antibody, biotinylated anti-rat Ig species-specific, streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech) and BCIP and NBT, as substrates (Shibata *et al*., 1999).

To detect the cytoskeletal protein, the same treated gels were blotted onto a PVDF membrane and treated with actin (N. 350), alpha-tubulin (N. 356), beta-tubulin (N. 357) and biotinylated goat antibody as the secondary antibody in the presence of 5 BCIP and NBT as substrates (Wako Chemicals). By using Pharmacia LKB Image Master DTS densitometer, the optical density of 49-kDa apyrase and cytoskeletal proteins were quantitated. Densitometry quantification of Western blot bands was made using Image Master. Measurements were done in triplicate and are presented as mean ± SEM.

Assay of ATPase and ADPase activity

Pea stems, leaves and roots were harvested, homogenised in cytoskeleton-stabilising buffer, filtered through two layers of Mira cloth and then centrifuged for 27,000 xg. The ATPase and ADPase activities were estimated as released phosphates, using ATP, ADP and AMP as substrates in the presence or absence of extracts of *E. arabicus*, as described by Moustafa *et al*., 2003. An assay mixture of 83.3 µL that composed of 100 mM Tricine-NaOH (pH 7.5), 10 mM $CaCl_2$, 10 mM substrate (nucleoside triphosphate, ADP, or AMP) was added to 1 µL from stems, leaves and roots diluted pellets with CSB then kept at 25°C for 15 min. To stop the reaction, 16.7 µL of 50% (v/v) TCA was added and then kept on ice. To measure the amount of released phosphate, 500 µL of ferrous sulphate-ammonium molybdate reagent was added to the treated and untreated samples. Protein content in treated and untreated samples was determined according to Bradford, 1976. Results are expressed as micromoles consumed per minute and per milligram of tissue (mean ± SEM).

Estimation the amino acids contents

According to the method of Moore and Stein, 1948, the total number of free amino acid was evaluated from dissected stems, leaves and roots of pea plant, either through germination in *E. arabicus* extracts or in DW. A citrate buffer of 10 ml was used to dissolve 16 mg of $SnCL_2$ mixed with 10 ml of ninhydrine solution. One ml of the prepared reagent was added to 200 ul of each plant extract and boiled for 20 minutes. Then, 5 ml of diluted solvent (an equal amount of distilled water and 95% ethanol) was added and kept for 15 min at 24°C. The absorbance was calculated using a spectrophotometer at 570 nm. Results are expressed as milligrams per gram of tissue (mean ± SEM).

Data analysis

The results were expressed as mean ± SEM. Data statistical analysis were carried out using Student's *t*-test and the results were considered significant at $P < 0.05$.

Results

Quantification of 49-kDa apyrase and cytoskeletal proteins

Densitometry quantification of each band found in the western blot graphs, using Image Master of 49-kDa apyrases and the sum of all cytoskeletal protein including actin, alpha and beta-tubulin, obtained are represented in Fig. 1 and Fig. 2.

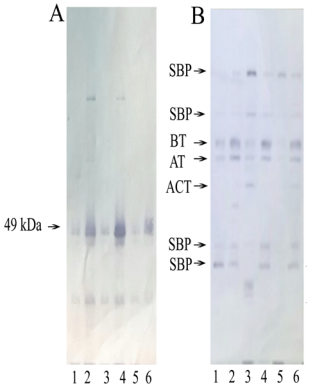

FIGURE 1. PVDF membrane of 49 kDa and cytoskeleton proteins. Pea seeds were imbibed and sown in DW in the dark for 72 hours. Dissected stems, leaves and roots grown in DW (lane1, lane3 and 5 respectively) and that grown in *E. arabicus* water extract (Lane 2, 4 and 6, respectively). Panel A: PVDF membrane probed with anti-apyrase antibody, antibiotinylated anti-rat Ig species-specific and streptavidin-alkaline phosphatase conjugate. Panel B: PVDF membrane probed with actin (N. 350), alpha-tubulin (N. 356), beta-tubulin (N. 357) and biotinylated goat antibody. SBP, streptavidin-binding proteins; BT, beta-tubulin; AT, Alpha-tubulin; ACT, actin-like protein.

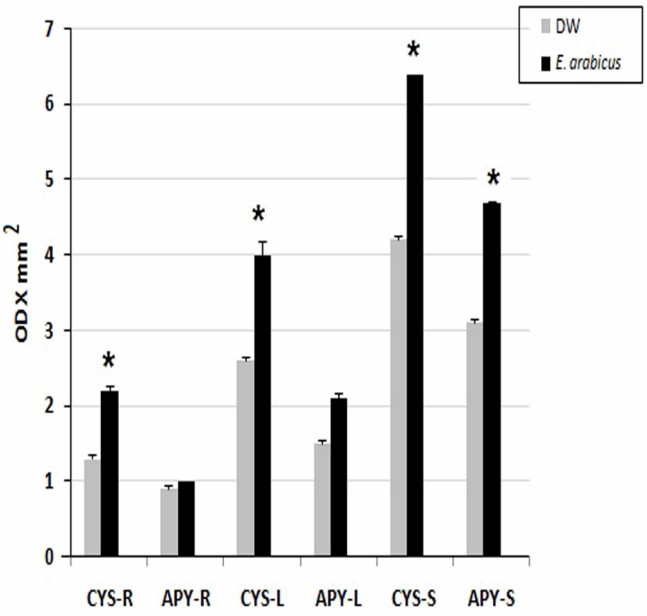

FIGURE 2. Optical density of 49 kDa and cytoskeletal proteins on PVDF membrane (Fig. 1). CYS-R, cytoskeletal proteins in roots; APY-R, 49 kDa in roots; CYS-L, cytoskeletal proteins in leaves; APY-L, 49 kDa in leaves; CYS-S, cytoskeletal proteins in stems APY-S; 49 kDa in stems. Each value is the mean ± SEM; *the mean difference is significant at $P < 0.05$ using Student's t test.

It was very clear that the sum of cytoskeletal protein and 49-kDa apyrases was abundant in the primary stems than primary roots and little in the roots. In all cases, the pea seeds grown in distilled water exhibited lesser amount of cytoskeletal proteins than those grown in *E. arabicus* extracts. The amount of cytoskeletal proteins increased progressively by 52.72% in the stem compared to roots and about 23.53% compared to leaves during the 72 hours of germination in distilled water. Notably, there were significant increases in cytoskeletal proteins found in roots, leaves and stems and in 49 kDa apyrase belongs to the stems (Fig. 2).

The primary stems had 49-kDa apyrases ranging from 34.78% to 55.00%, more than leaves and root progressively, during the 72 hours of germination in distilled water. Stems of pea seeds grown in *E. arabicus* extracts manifested 23.06% more cytoskeletal proteins than that in leaves and 49.18% more that roots while 49-kDa apyrases exhibited an increase of 37.76% compared to leaves and 64.66% compared to root throughout their germination in *E. arabicus* water extracts. The amount of 49-kDa apyrase in pea root, grown in *E. arabicus* water extracts, was more than that grown in DW by approximately 5.63% while the amount in leaves and stems increased by about 16.89% and for stems by 20.43%. Cytoskeletal proteins for pea seeds grown in *E. arabicus* water extracts increased in range between 20.64%, 21.09% and 25.74% for stems, leaves and roots, respectively, than that grown in DW thorough 72 h of germination.

Apyrase activity

For more obvious determination in the increment in ATPase activities associated with germination because of germination in *E. arabicus* water extracts, the homogenates with 49-kDa apyrase, which were obtained from various tissues, were assayed against ATP, ADP and AMP, as shown in Fig. 3.

No hydrolytic activity was observed against AMP from all tested samples. Extracts of pea stems were germinated for 72 hours showed virtually the highest activity against ATP and ADP substrate, whereas the leaves tissue was intermediate, and the roots had the lowest activity. There was significant difference for ADPase and ATPase between the plant treated with *E. arabicus* extract and DW throughout the treatment period in the stems and leaves parts. Estimation of the differences between ATPase activity and ADPase growing in DW was found to be 23%, 30.24% and 45.30% in roots, leaves and stems, respectively, while 42.516% for roots, 52.02% for leaves and 39.90% for stems in *E. arabicus* water extracts. ADPase activities were higher in the pea stems growing in the DW by 46.85%.and 41.48% than leaves and roots respectively while those growing in *E. arabicus* water extracts were higher by 30.10% for leaves and by 48.00% for roots.

Also, ATPase activities were higher in the pea stems growing in the DW by 39.40% for leaves and 45.03% for root while those growing in *E. arabicus* extracts were higher by 34.03% for leaves and by 49.33% for roots. ATP exhibited an increased efficiency rate of pea seedling growing in *E. arabicus* by 17.89% for roots, 42.43% for leaves and 40.54% for stems, and ATPase exhibited 20.94% for roots, 39.30% for leaves and 31.77% for stems.

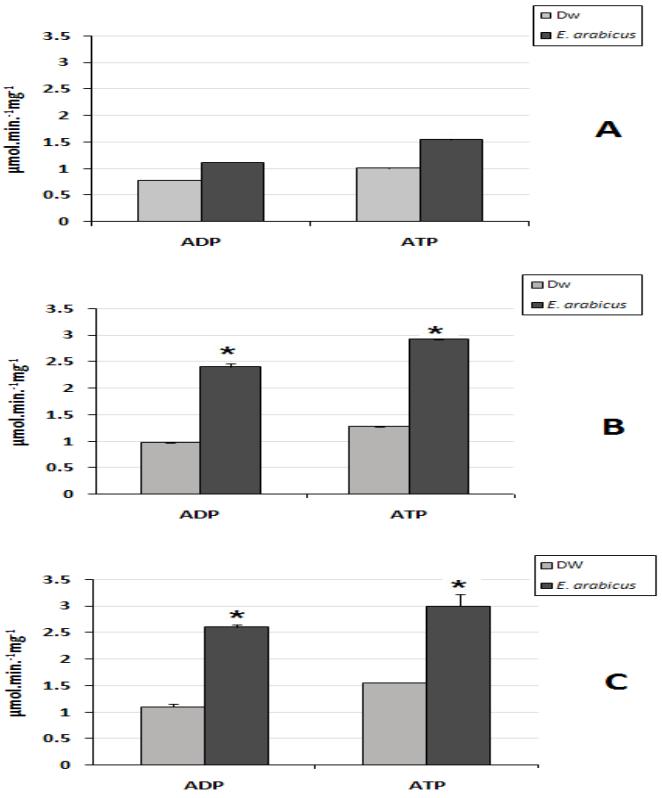

FIGURE 3. ATPase and ADPase activities on germination of pea seeds in distilled water and in *E. arabicus* water extract. Panel A; Roots; Panel B, Leaves; Panel C, Stems. Each value is the mean ± SEM; *the mean difference is significant at $P < 0.05$ using Student's *t* test.

The close correlation between the efficiency of three tissues against the two substrates strongly argues that the majority of ATPase and ADPase activities are due to the active components present in the extracts of *E. arabicus*.

Amino acid contents

Amino acids are candidate molecules that mainly contribute to the metabolic process during the pea seedling germination under specific environmental conditions. Therefore, they were categorised into 3 main components, stems, leaves and roots, and these were determined (Fig. 4). Under the control condition, the amino acids varied depending on which part was investigated and affected by the extracts of *E. arabicus* especially for stems and leaves as they found to be had significant results (Fig. 4 Panel B and C).

Under the DW irrigation condition, the amino acid content greatly increased by more than two or three fold in stems than in leaves and roots as well as under the effect of the extracts of *E. arabicus*. Amino acids increased in the stem of peas seeds growing in the DW by 38.46% for leaves and 47.45% for root while those growing in the *E. arabicus* extracts increased by 22.66% compared to leaves and 32.19% compared to roots. *E. arabicus* extracts increased the amino acid contents in all tissues in the order of root (13.37%), leaves (23.68%), and stems (51.15%).

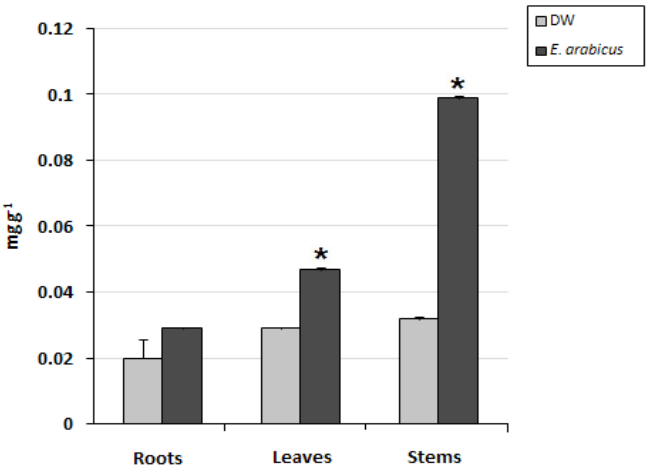

FIGURE 4. Amino acids contents in pea stem, leaves and root grown with distilled water (DW) or *E. arabicus* water extract. Each value is the mean ± SEM; *the mean difference is significant at $P < 0.05$ using Student's *t* test.

Discussion

Link between 49-kDa apyrase and cytoskeletal proteins

Many plant extracts were considered as an important factor that could affect the plant physiological characteristics (Ali *et al.*, 2015; Hernández-Herrera *et al.*, 2016; Prithiviraj, 2008). The allelopathic effect of the plant extract has been reported to depend on the species and the stage of development (Moustafa *et al.*, 2016; Agarwal *et al.*, 2002). In the present research, we examined the possible impacts of *E. arabicus* water crude extract on pea seedlings, which were under 72 h of germination-various parts including stems, leaves and roots. Pea's seeds grown in water had a significant raise in the amount of 49-kDa apyrase and cytoskeletal protein in the shoots system while the degree of assimilation was low in roots.

The general increase in the rate of assimilation in stems, leaves and roots in 49-kDa apyrases was mainly linked with the increase in the cytoskeletal proteins. In a typical case, and by using 2D-PAGE and Western blotting to examine the rate of expression of various isotypes of apyrase (EC 3.6.1.5) during the initial growth of pea (*Pisum sativum* L. var. Alaska) seedlings, it was found that 49-kDa apyrase began to appear 16 hours after germination and increased through germination time and was higher in stems than leaves and roots (Moustafa *et al.*, 2003).

Moreover, a fraction from the homogenates of Alaska peas (*Pisum sativum* L.) under germination for 30, 45 and 69 hours, respectively, were tested for the status of the cytoskeletal protein in the embryo and its different parts, and it was found that by 69 hours, abundance of cytoskeletal protein in different fractions of stems was higher than that of roots and prophyllus and that there were very little amounts of all types of cytoskeletal proteins in the primary roots (Moustafa, 2009).

Furthermore, an increase in 49-kDa apyrase and cytoskeletal proteins were observed while pea seeds grown in the *E. arabicus* water crude extract especially for cytoskeletal proteins found in all parts and in 49 kDa apyrase found in the stems. The active molecule in the *E. arabicus*, grown in EL Souda Mounatins, KSA, was identified previously (Moustafa *et al*., 2018). It is necessary to characterise such inorganic or organic molecules (e.g., micronutrient, lipids, lignin, silicate, hormone, etc.) that might be abundant in *E. arabicus* crude water extract than DW itself.

Effect of plant extract as natural fertilizers has been reported in several plants-*Oryza sativa* (Ilori *et al*., 2007), some varieties of wheat (Agarwal *et al.,* 2002) and common bean (*Phaseolus vulgaris* L.) (Al-Watban and Salama, 2012). These active components may have a role in the metabolic process in leaves, stems and roots of pea's seedling. They may have a role in the metabolic process in regulation of leaf respiration and alternative pathway activity. For example, the rate of plant respiration is organised by many factors such as the availability of substrates and their type, adenine diphosphate and carbohydrate (Krapp *et al*., 1991; Azcón-Bieto *et al*., 1994; Gonzàlez-Meler and Siedow, 1999).

It was also reported that the type of substrate and available active components could affect the photosynthesis rate as well as the cellular ATP utilisation rate associated with transport and biosynthetic processes and general cellular maintenance (Beevers, 1970; Fader and Koller, 1984).

ATPase and ADPase activities

ATPase and ADPase activities are considered as measuring tools of plant metabolic activities, playing a vital role in many processes. For example proteins transportation are activated by electrochemical gradients of protons through the plasma membrane due to the action of plasma membrane H^+ pumps fuelled by ATP (Palmgren, 2001).

Herein, we analysed both ATP and ADP, as the apyrase, which is considered as a highly active ATP-diphosphohydrolase, catalysed the sequential hydrolysis of ATP to ADP to AMP, yielding inorganic phosphate. Figure 4 shows that the ATP and ADPase values of each part are positively correlated to the germinations in the *E. arabicus* aqueous extracts and significantly higher in stems and leaves compared with the roots tissue. This again indicates that the growth rate basically depends on the active compounds in the *E. arabicus* extracts. Furthermore, the variation in each part, whereas ATPase and ADPase are more in stems than leaves and roots, ensures the correlations among ATPase, ADPase, 49-kDa apyrase and total cytoskeletals proteins.

Previously, a similar conclusion was drawn on the relationship between the expression of different isotypes of apyrase when imbibition, germination stages and initial growth of pea was investigated using 2D-PAGE and Western blotting, and it was found that stems contained all five isotypes, with p*I* 6.0, 6.3 and 6.6, isotype than the leaves; whereas, less amounts of all isotypes were found in the roots, concluding that ATPase activity mainly results from the 49-kDa apyrase. ATPases may be responsible for the different functions in each part of pea seedlings, but the real function needs more investigation. By using an electron microscope, the most pronounced staining part for ATPase was located at the plasmalemma for either wheat coleoptiles or pea stems with regard to the postulated functions of ATPase activity in elongation of plant cell and in proton pumping (Hall *et al*., 1980).

Amino acids

It is well known that amino acids have crucial functions throughout plant life. Apart from their function during the biosynthesis of the protein, it showed several other metabolic pathways during signalling processes (Hildebrandt *et al*., 2015).

In general, the recorded amount of amino acids differ strongly in stems, leaves and roots and change dynamically depending on the substrate of the growing conditions. The amount of amino acid is lesser in all pea seedling parts that grow in the water than those grown in the *E. arabicus* extracts. There are significant activity in the stems and leaf extract than roots proofed the importance of both *E. arabicus* extracts and amino acids to the various metabolic activity. As it was reported that many amino acids were found to be precursors for the biosynthesis of secondary metabolites such as glucosinolates, which can be generated from methionine, alanine, aromatic amino acids and branched-chain. Also, it was interpreted that plants that grow in substrate have no source of carbon, e.g., carbon starvation, or in distilled water only; then the amino acid could be subjected to the degradation to establish the energy condition of plant cells under germination in certain physiological state (Halkier and Gershenzon, 2006).

In addition, the energy requirements of the young seedling have to be covered by amino acid oxidative process and degradation of other storage materials, such as starch and fatty acids, until the photosynthetic apparatus is completely functional (Galili *et al*., 2014). Amino acids, under nutrient limitation source, are recycled and used for the synthesis of specific proteins that are required. Also, Hannah *et al*., 2010, Timm *et al*., 2012 and Häusler *et al*., 2014 found that in a non-meristematic parts, amino acids for protein synthesis can mainly be provided only by a protein turnover that acts as signalling molecules, precursors for the synthesis of phytohormones and for secondary metabolites activity with signalling function. Others showed that inorganic salts that are found less in water and more in plant extract initially contribute nitrogen that is rapidly linked to amino acids in the root or mature leaf tissue. Therefore, development of leaves, meristematic tissues and various reproductive organs should import amino acids to establish their growth and development (Ortiz-Lopez, A, 2000; Bush, 1999).

As a consequence, it could be expected that the regulation and the suitable amount of amino acid, generated by various parts of a pea seedling, can be highly organised by specific regulators found in plant extracts of *E. arabicus* extracts. These parameters (amino acids. ATPase, ADPase, 49-kDa apyrase and cytoskeletal proteins) can be used as a convenient evidence to distinguish the positive and non-positive extracts of various plants as natural fertilizers to plant growth. In conclusion, biochemical characterisation of the 49-kDa, cytoskeletal proteins, ADPase and ATPase activities and amino acid associated with germination of pea seeds in 7% *E. arabicus* aqueous water extracts have indicated

that the plant can play a crucial physiological role in pea plants and perhaps in other plants.

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