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FUNCTIONS OF FOLIC ACID (VITAMIN B9) AGAINST CYTOTOXIC EFFECTS OF SALT STRESS IN *HORDEUM VULGARE* L.

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Abstract

The function of vitamin B9 (folic acid) on mitotic activity and chromosome behaviors in meristematic cells of barley (*Hordeum vulgare* L. cv. 'Bülbül 89') seeds germinated at different salt concentrations were studied. The prohibitory impact of salinity on mitotic index significantly increased in parallel with increase of salt concentrations as compared to control group. The mitotic inhibition was 33.3% at 0.35 M salt concentration. Furthermore, the mitotic inhibition reached the lowest value (46.6%) after treatment with folic acid alone. The frequency of chromosome aberrances in meristematic cells of barley seeds treated with folic acid alone demonstrated a marked increase (approx. 32%) compared to the control seeds. The frequency of aberrances were gradually increased with increasing salt concentrations. In addition, folic acid pretreatment was statistically ineffective in recovering of the unfavourable effect of different salt concentrations on mitotic activity while it reduced the harmful effects of salinity on chromosome aberrances particularly at high salt concentrations.

Key words: Barley, Chromosome abnormalities, Folic acid, Mitotic index, Salinity, Toxicity.

Introduction

Salinity is a common worldwide environmental challenge and it is one of the major problems that limit agricultural production. About 20% of the cultivated land, which accounts for over 6% of the world total area, is threatened by salinity (Anon., 2015). Vanquishing of salt stress is an important subject for increasing plant growth and productivity. Plants have developed complex mechanisms for recovering salt stress and in this way contributed to the adaptation against to osmotic and oxidative stresses caused by salinity. All this mechanisms assist to stabilize of detrimental effects of high concentration of salts in cellular activities (Ashraf & Harris, 2004; Munns, 2002). Moreover, a lot of researchers accept that the best way to continue with cultivation would be via pyramiding various physiological characteristics. However, it is even so difficult to understand the mechanisms behind it in order to be able to cope with salinity (Zhu *et al*., 2016).

One of most important way to reduce the harmful effects of salinity on plant cultivation is to produce plant varieties resistant to salinity For this reason, researchers have used various plant hormones, growth regulators and some plant extracts to diminish or extinguish harmful effects of salt stress on seed germination (Duan *et al*., 2008), seedling growth (Çavuşoğlu *et al*., 2016; Khan *et al*., 2019), oxidative stability (Farheen *et al*., 2018), mitotic index and chromosomal aberrations (Tabur & Demir, 2010 a, b; Maraklı *et al*., 2014; Mahfouz & Rayan, 2017). Moreover, numerous researchers contributed to amelioration of the adverse effects of salinity by some vitamin treatments (Shalata & Neumann, 2001; Barakat, 2003; Bassuony *et al*., 2008; Emam & Helal, 2008; Mohsen *et al*., 2014). In fact, plants do not synthesize vitamins for the benefit of only animals because they play the same essential role for the plant's own metabolism. Requirements for vitamin concentration of plant cells are varied according to the plant species and type of culture (Abrahamian & Kantharajah, 2011). Most vitamins act as cofactors for many enzymes (Smith *et al*., 2007). Folate known as folic acid (vitamin B9), is an essential member of vitamin B complex. Vitamin B9 cannot be synthesized by humans and animals; herbal foods is the main source of this essential vitamin. Also, it is known that folic acid served as a cofactor for enzymes that are essential for RNA and DNA synthesis and have stimulating effect on plant growth and development (Rosenquist *et al*., 1996; Scott *et al*., 2000). Vitamin B9 is important in periods where cell division is rapid and in growth stage especially due to helps to produce and maintain new cells in mammals and plants. Insufficiency of folic acid during cell division and growth causes to lose their ability to division and proliferation of cells. This case is slow down nucleic acid synthesis and interrupt cell division (Globalherbalsupplies, 2018). In addition, folic acid aids protect metabolism and DNA of plants however folic acid distrupt easily in plants especially under extrem lighting, so it is suggested folic acid as supplemental feed to plants (Manicbotanix, 2015).

The stimulating effect of folic acid on plant growth and development by increasing the mitotic division is known for a long time (Jacobson, 1954; Gambonnet *et al*., 2001; Jabrin *et al*., 2003; Hillis *et al*., 2011). At the same time, folic acid increases the root elongation, germination percentage and stem width at the beginning of germination (Gambonnet *et al*., 2001; Burguieres *et al*., 2007); seed weight, amount of chlorophyll in leaves (Stakhova *et al*., 2000; El Saidy *et al*., 2011) and synthesis of DNA and RNA (Kamen, 1997); and also have the potential to function as natural antioxidants and growth regulators (Mc Cue *et al*., 2000).

As mentioned above, there are numerous reports related to effects on seed germination, seedling growth, enzyme activity, protein synthesis and some metabolic changes of many vitamins, including folic acid in plants. However, there is no extent literature about effects of folic acid on mitotic activity and chromosome aberrances in normal and salt stress conditions. Therefore, this work was designed to test the effectiveness of folic acid in the adjusting of harmful effects of salinity on the mitotic activity and chromosome aberrances in root meristem cells of barley seeds during mitotic division.

Materials and Methods

The barley seeds were obtained from the Field Crops Research Institute, Ankara, Turkey. The barley seeds were sterilized with 1.0% NaClO for 10 min, washed under running tap water, and soaked in constant volumes of distilled water (control, C) and folic acid (50 µM, micromolar) for 24 h at 20 ± 1°C. Seeds almost equally large were placed to Petri dishes covered with filter papers moistened with 7 ml of distilled water or different NaCl concentrations (0.0, 0.25, 0.30 and 0.35 M, molar). Then, Petri dishes were transferred for germination to an incubator set to room temperature for 3-5 days. Salt levels hindering germination of seeds on a large scale and the proper concentration of folic acid used were adjusted to test the salt sensitivity of barley seeds. 50 µM concentration of folic acid were the most accomplished level in alleviation of the salt inhibition at the germination.

After several days, the root tips reached to 0.5-1 cm were excised, preterated with a saturated solution of paradichlorobenzene for 4 h at 20ºC, fixed with asetic alcohol (3:1) for 24 h, and stored in 70% alcohol at 4ºC until required. Then, root tips were hydrolyzed in 1 N HCl at 60ºC for 17 min, stained with Feulgen for 1 h, and squashed in 45 % acetic acid (Sharma & Gupta, 1982). After one day, slides were made permanent by mounting in balsame. The best mitosis phases and aberrances were observed in permanent slides and photographed (100X) with a digital camera (Olympus C-5060) mounted on an Olympus CX41 microscope.

To detect mitotic index, approximately 3000 cells were scored in both control and in treated groups. Chromosome aberrances were calculated for each concentration as the percentage of 300 dividing cells was calculated. Percentage of the mitotic inhibition was also calculated using the equation as follow.

$$\text{Mitotic inhibition} = \frac{\text{Mitotic index in control} - \text{Mitotic index in treated}}{\text{mitotic index in control}} \times 100$$

All experiments were repeated three times. Statistical evaluations of obtained data were performed using the SPSS 14.0 program and Duncan's multiple range test (Duncan, 1955).

Results

Effects of exogenous folic acid on mitotic activity in normal and saline conditions: As compared control, mitotic index of seeds germinated in distilled water after treating with folic acid showed a significant decrease. In the other words, the mitotic index of control seeds performed better than the seeds treated with folic acid. The mitotic inhibition value of 50 µM folic acid application was 46.6%. The value is statistically significant.

Moreover, the increase in the salt concentrations remarkably reduced the mitotic index. 50 µM folic acid pre-treatment could not alleviate the negative effect of salt stress on mitotic activity. In briefly, effect of folic acid pre-treatment on mitotic index in response to increasing salt levels showed almost close values to each other in three salt concentrations (Table 1).

Effects of exogenous folic acid on chromosome behaviours in normal and saline conditions: Mitotic phases in root meristem cells of barley seeds in control group were observed as normal (Fig. 1). The percentage of chromosome aberrance in root meristem cells was increased approximately 32% with folic acid alone pretreatment. Moreover, a result of mitosis scans, a wide range of chromosome aberrances revealed together with increasing salt concentration. However, 50 µM folic acid application exhibited a wonderful performance in diminishing the harmful effects of salinity to chromosome aberrances particularly at high salt concentrations (0.30 and 0.35 M NaCl). The mitotic index, mitotic inhibition and chromosome aberrance values provided from the control and treated seeds are given in Table 1.

Given all the applications studied in general, considerable chromosome aberrances, such as micronucleus, uncoilling chromosome, irregular anaphase, bridges, laggards, alignment anaphase, fault polarization in anaphase and vagrant chromosome were observed (Fig. 2a-l). The most common among aberrances recorded in all applications were bridges of anaphase (Fig. 2f, g) and alignment anaphase (Fig. 2ı, j). Particularly, some abnormalities such as bridges of anaphase and laggard chromosomes (Fig. 2f, g, h), viewed in meristem cells of seeds treated with folic acid were noticeably more than other abnormalities. In samples germinations in different salt concentrations were recorded along with quite a few presence of micronucleus (Fig. 2a).

Table 1. The values of mitotic index, chromosome aberrance and mitotic inhibition in root meristem cells of barley seeds germinated in distilled water and various NaCl concentrations after 50 µM folic acid pretreatment

NaCl (M, molar)	Folic acid (FA) (50 µM)	Mitotic index (%)	Chromosome aberrance (%)	Mitotic inhibition (%)
0.0	Control	*0.15 ± 0.32^{b}	0.00 ± 0.00^{a}	-
	FA	0.08 ± 0.00^{a}	31.4 ± 4.6^{bc}	46.6
0.25	Control	0.13 ± 0.13^{ab}	25.7 ± 7.5^{b}	13.3
	FA	0.10 ± 0.01^{a}	59.0 ± 2^{d}	33.3
0.30	Control	0.11 ± 0.01^{a}	76.5 ± 2.8^{e}	26.6
	FA	0.10 ± 0.01^{a}	45 ± 4.7^{cd}	33.3
0.35	Control	0.10 ± 0.01^{a}	83.5 ± 7^{e}	33.3
	FA	0.10 ± 0.01^{a}	50.8 ± 8.2^{d}	33.3

*Shows values with insignificant difference ($p<0.05$) for each column shown with same letters (± standart deviation)

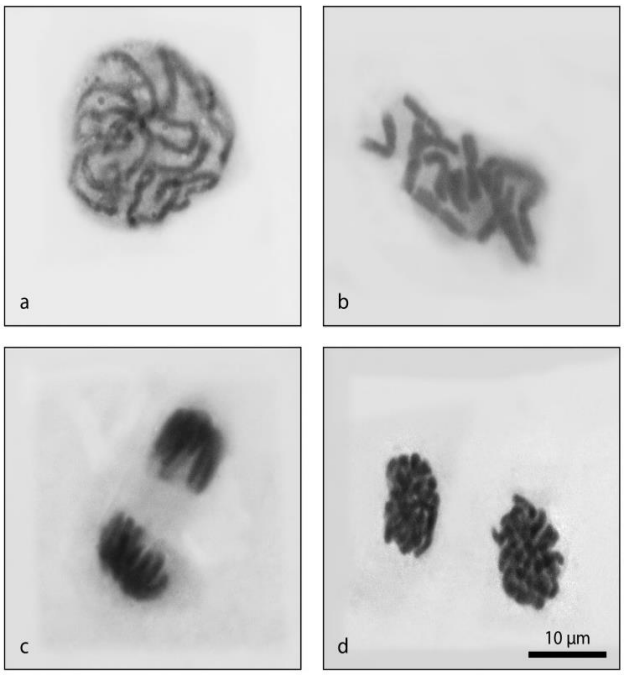

Fig. 1. Normal mitotic phases in root meristem cells of barley seeds germinated in distilled water without any treatments. (a) prophase, (b) metaphase (2n = 14), (c) anaphase, (d) telophase. Scale bar = 10µm.

Discussion

Cytogenetic responses of exogenous folic acid in non-stress conditions: Until now, there are no studies relating to the effects of folic acid on the mitotic activity and chromosome aberrances in non-stress conditions in plants. For this reason, we have examined at first whether folic acid is impressiving these parameters in non-stress conditions or not. According to our findings the mitotic index in root meristems of seeds exposed to folic acid pre-treatment for 24 h in non-stress conditions was decreased approximately two folds compared to those of in distilled water. The limit of mitotic inhibition of this proceeding was 46.6%. In the other words, 50 µM application of folic acid displayed an inhibitor impact on the mitosis. In this case, folic acid might have acted as a stimulator by hindering the synthesis of protein necessary for the mitosis and by slowing down cell cycle. Furthermore, percentage of chromosome aberrances was increased three fold with dose of folic acid used in the present work. If so, it would not be wrong to say that some abnormalities might have been resulted from this stimulator (Table 1). Thus proving that no exogenous supplement of any stimulator in non-stress conditions is required.

Cytogenetic responses of exogenous folic acid in salt stressful conditions: Mitotic index (MI) is one of the cytotoxic assays often used in the studies of biotic and abiotic stress. Mitotic index scores may be useful to determine the cytotoxic effect of any test compound (Fernandes *et al*., 2007). The cytotoxic and repressive effects on the mitotic activity of salinity, which is one of the most important abiotic stresses, are known for a long time (Lutsenko *et al.*, 2005; Radic *et al.*, 2005; Tabur & Demir, 2010a, b; Kielkowska, 2017). Radic *et al*., (2005) reported that in root meristem cells, increase of salt concentrations caused total inhibition of mitotic process and increase chromosome abnormalities. Tabur & Demir (2010 a, b) suggested that salinity, especially at high levels had chromotoxic actions and caused to decrease mitotic index. A recent study has shown that salt has caused cytotoxic effects on meristematic cells in both modern wheat species and the ancient einkorn wheat (Pekol *et al.*, 2016).

Findings in the present work indicated that mitotic index of barley seeds was reduced remarkably with increasing salt levels and caused higher number of chromosomal abnormalities. The mitotic inhibition at the highest NaCl concentration tested was recorded as 33.3% and a considerable drop in the mitotic index. These findings point out that salt causes mitostatic effect at this treatment. Mitotic inhibition was due to the arrest of cells at G1 phase by suppressing DNA synthesis (Schneiderman *et al*., 1971), or block in G2 by preventing the cells from entering mitosis (Van't Hof, 1968). Additionally, as compared to control, rises in salt level created a drastical increase in the chromosome aberrances. For example, this value was 83.5% at 0.35 M NaCl, the highest concentration tested (Table 1). For the correct segregation of mitotic chromosomes it is essential that sister chromatids connect to microtubules spreading from opposite spindle poles. Connecting sister chromatids is stochastic process, prone to make error and can cause chromosome irregularities (Rieder & Salmon, 1998).

The mitotic index of barley seeds germinated in different salt levels after the folic acid pretreatment could not reached to those of control seeds germinated in distilled water. Moreover, as compared to own controls, folic acid pretreatment was statistically ineffective in alleviating of the undesirable effect of different salt levels on mitotic activity, even if a slight increase at low salt levels numerically. However, this vitamin exhibited very successful performance in ameliorating of the harmful effects of salinity on chromosome aberrances according to own controls, particularly at high salt concentrations (0.30 and 0.35 M NaCl). This result demonstrates that folic acid has the protective role against salt damages in root tip meristems of barley during mitosis division. These protective effects might have also been essentially originated from factors such as species of plant, the developmental stage of plants, resistance to stress and period of the treatment.

All of these mitoclassic impacts such as irregular anaphase, fault polarization, alignment anaphase, vagrant chromsomes and bridges might have been largely resulted from spindle difunction, and these disorganizations constitute an important portion of chromosome abnormalities. As Tabur & Demir (2010 b) also stated, the bridge formations in anaphase and telophase might had been the result of inversions. The micronuclei are probably the result of vagrant chromosomes and fragments (Briand & Kapoor, 1989). The laggard of chromosomes originates from a weak mitotic impress. It known that salt stress, particularly NaCl caused to many c-mitotic reactions (Fiskesjö, 1997). Therefore, increasing salt concentrations may give rise to the formation of laggard chromosomes at high rates. Irregular chromosome contractions may lead to uncoiling chromosomes in prophase and metaphase cells.

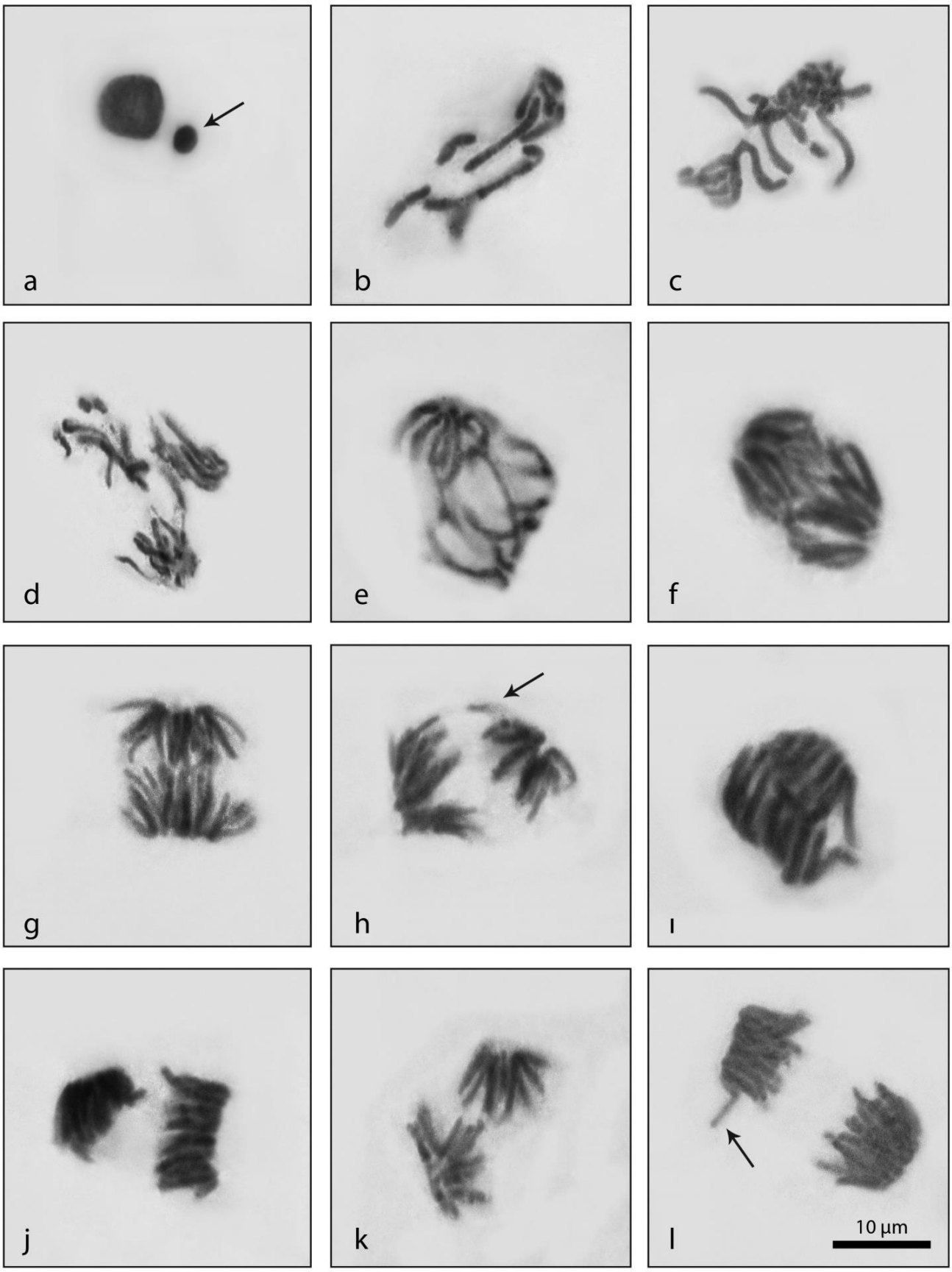

Fig. 2. Chromosome aberrances in mitotic phases in root meristem cells of barley seeds germinated in distiled water and various NaCl concentrations after pretreatment with folic acid. Folic acid concentration: 50 µM (micromolar). NaCl concentrations: 0.25- 0.30- 0.35 M (molar). (a) micronucleus (arrow), (b, c) uncolling chromosomes, (d, e) irregular anaphase, (f, g) anaphase bridges, (h) lagging chromosome in anaphase (arrow), (ı, j) alignment anaphase, (k) fault polarization in anaphase, (l) vagrant chromosome in telophase (arrow). Scale bar = 10 µm.

Conclusions

Present findings demonstrates that alone folic acid or salty conditions may disrupt the reactions relating to mitotic activations. Both NaCl and alone folic acid caused the reduction of the mitotic index in barley root meristems and significantly increased chromosome aberrations. However, 0.35 M salinity displayed more severe cytotoxic effect than folic acid alone since it caused to the highest percentage of aberrations. In parallel with the increasing of the salt concentrations, folic acid pretreatment was not found enough successful on mitotic activity but it exhibited much powerful performance on chromosome aberrations. Hence, we can say that exogenously folic acid at suitable doses might help to ameliorate the chromotoxic effects caused by the salinity on mitotic processes in the course of germination. In order to elucidate the cytogenetical role of folic acid, it is much important and requiste to reveal the influence of its biosynthetic inhibitors on chromosome actions during cell division in various plant species. Folic acid (vitamin B9) is a very essential vitamin that enhances and protects metabolic processes particularly those involved in DNA synthesis and gene expression. In the future, working a wider range of the simultaneously effects of different vitamins and plants is needed for an enhanced feasibility outcome. Doubtless, our study will be a baseline to similar work in the future.

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