Abstract—An efficient protocol has been established for the regeneration of Cardamine hirsuta. The objective of this study was to evaluate the effect of types and concentrations of cytokinins on in vitro regeneration of C. hirsuta, an important medicinal and edible plant. Nodal explants of C. hirsuta were cultured in Murashige & Skoog (MS) media containing kinetin or zeatin singly at concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0 mg/L. MS medium devoid of plant growth regulators served as control. The growth parameter observed including number of shoots, leaves, and roots. 2.0 mg/L zeatin induced the highest number of shoots and leaves. 1.0 mg/L kinetin-treated explants showed the highest formation of roots. From the results obtained, it was learnt that 2.0 mg/L zeatin was a more effective cytokinin compared to kinetin for in vitro regeneration of C. hirsuta as it induced the highest shoot and leaf growth while minimizing root formation. Concentrations of zeatin above optimal level not only caused stunted growth, but also resulted in hyperhydration. In conclusion, in vitro regeneration can serve as an alternative propagation method for C. hirsuta.

Index Terms—Cardamine hirsuta, in vitro regeneration, cytokinin, zeatin, kinetin

I. INTRODUCTION

Cardamine hirsuta L., commonly known as hairy bittercress, is a herb that belongs to the genus Cardamine, and family Brassicaceae (mustard) [1]. C. hirsuta is a medicinal plant that has demonstrated antipyretic and diuretic properties which are important in treating urinary and bladder inflammations, dysentery, and white discharges [2]. Besides, it can also be used to cure insomnia and toothache [3]. It is a potherb that can be eaten raw or cooked. The edible parts include flowers and leaves, which are commonly added in salad for their peppery taste [4]-[6].

In vitro regeneration (micropropagation) is a plant tissue culture protocol to produce many identical copies of a plant from its tissue segment via in vitro techniques [7]-[9]. In general, a fragment of a germ-free plant tissue or explant from the “mother plant” is cultured in an axenic nutrient medium [8]. The medium composition and physical conditions are manipulated to direct the desired pattern of growth and reproduce the whole plant, where the newly produced plants are clones of the “mother plant” with identical genetic make-ups [8].

The development of a desired tissue is caused by the action of plant growth regulators (PGRs) or plant hormones [9]. Cytokinin is a type of PGR that functions to induce cell division and protein synthesis, leading to many plant processes such as delay of aging, chloroplast formation, uptake of resources, wound healing, and vascular growth [10] [11].

Cytokinin primarily stimulates development of shoots and prevents the development of roots. When applied to a shoot culture medium, apical dominance which is controlled by auxin is inhibited, thus leading to lateral bud shooting [7] [12]. Axillary bud formation is an effective pathway of plant tissue culture initiated by cytokinins [10].

In vitro regeneration technique is suggested to be carried out to enable commercial exploitation and large scale production of C. hirsuta for its medicinal properties. The aim of this study was to study the effect of different types and concentrations of cytokinins on in vitro regeneration of C. hirsuta from nodal explants.

II. MATERIALS AND METHODS

A. Plant Material

Pre-cultured C. hirsuta was obtained from Biotechnology lab of INTI International University as the source of explants.

B. Effect of Types and Concentrations of Cytokinin on the Growth of C. hirsuta

Nodal segments of approximately 1.0 cm long were isolated from in vitro plantlets of C. hirsuta using sterile scalpels in laminar air flow cabinet. Each excised stem was placed vertically on culture medium supplemented with either kinetin or zeatin at concentrations of 1.0, 2.0, 3.0, 4.0, or 5.0 mg/L. Randomization of culture vials was done prior to culture initiation to ensure non-biased culturing. The growth of plantlets in terms of number of shoots, number of leaves, and number of roots was observed weekly for 8 weeks continuously.

C. Statistical Analysis

Means among replicates was obtained using Microsoft Excel 2010. Data collected were statistically analyzed by one way analysis of variance (ANOVA) with 95% confidence level.

III. RESULTS
A. Effect of Types and Concentrations of Cytokinin on the Growth of C. hirsuta

At the end of observation period (week 8), plantlets treated with different types and concentrations of cytokinins were compared with control plantlet which was not treated with any plant growth regulators (Fig. 1).

At the end of this study, abnormal morphologies of plantlets were also observed, which include hyperhydricity and stunted shoot growth (Fig. 2). These phenomenons were seen in several plantlets treated with zeatin at high concentrations (3.0 mg/L - 5.0 mg/L). Hyperhydrated plantlets appeared glassy and brittle while plantlets with stunted shoots appear compact, with multiple shoots that were short, and small leaves.

IV. DISCUSSION

A. Effect of Types and Concentrations of Cytokinin on the Growth of C. hirsuta

In this study, control medium that was not supplemented with cytokinin responded to growth. However, it resulted in formation of a single shoot. The growth of shoot was due to the presence of endogenous cytokinin that naturally existed in the plant. However, newly excised explants usually require more time to manufacture cytokinins, and the absence of exogenous cytokinin made it more difficult to undergo shoot formation. This confirmed that exogenous cytokinin is required in culture medium to achieve the adequate shoot formation [13]. Hence, without the inclusion of cytokinin in culture, explants will still be able to form shoots but with lower yield. This explained the formation of a single shoot in the control medium. On top of that, high cytokinin levels generally induce multiple shoot formation by inhibiting shoot apical dominance which leads to formation of lateral shoots [10].

It was observed that the development of shoots was consistent with leaves. The highest levels of shoot and leaf numbers were observed in plantlets treated with zeatin, with an optimal concentration of 2.0 mg/L (31 shoots and 52 leaves). In accordance to the results obtained, a study done by [14] also indicated that zeatin induced higher shoot proliferation than kinetin. [15] stated that zeatin provided higher shoot proliferation compared to BAP and kinetin. A study of effects on PGRs on Labisia pumila var. alata demonstrated a higher formation of shoots in media containing zeatin compared to media containing kinetin [16]. Natural cytokinin, zeatin, promoted higher rate of growth and survival of shoot cultures of Asparagus plumosus compared to synthetic cytokinins, kinetin and BAP [12]. A similar result was obtained in plants of the Ericaceae family, in which zeatin induced higher shoot proliferation rate than other synthetic cytokinins [12]. High number of multiple shoot formation in zeatin-treated medium was due to its ability to induce rapid cell division in the meristem of the nodal segment of C. hirsuta compared to kinetin and BAP [17]. [18] demonstrated that formation of leaves was slow in Arabidopsis plants without cytokinin supplement, and the number of leaf cells reduced tremendously. [19] demonstrated that expression of cytokinin gene (IPT7) in tomato leaves increased the number of leaflets. These signified that cytokinin acts as a positive regulator in both shoot and leaf development.

Although cytokinin inhibits root formation [7] [20], in rare cases, it had been shown that cytokinin-treated plants...
to have positive effects on root development [21]. A study done by [22] showed positive effect of kinetin on rooting of *Matthiola incana*. Root formation of tobacco stem segments had been seen in medium with kinetin [23]. [24] demonstrated formation of pseudonodules initiated by exogenous kinetin on tobacco roots. In this study, zeatin-treated plantlets had roots too, but the number of roots was much lesser (0-4 roots) as compared to kinetin-treated plantlets (5-15 roots). This indicated a higher activity of cytokinin in zeatin-treated explants which resulted in high shoot formation and minimal root formation. Compared to kinetin, zeatin showed a more significant activity of cytokinin, as the antagonistic interaction between shooting and rooting was clearly observed in zeatin-treated plantlets. Apart from that, plants with low cytokinin activity generate more roots. Plants with larger root systems are generally unfavorable for growth of the plants as factors such as intake of minerals and water would be higher, thereby limiting for growth of the plants as factors such as intake of minerals and water would be higher, thereby limiting growth. Therefore, zeatin is a more efficient and effective cytokinin than kinetin in *C. hirsuta*.

The shoot proliferation in cytokinin-treated plantlets increased as the concentration of PGR increased due to rapid cell division triggered by the exogenous cytokinin. Concentrations of zeatin and kinetin above optimal level resulted in reduced number of shoots as the highly concentrated cytokinin turned toxic to the plantlets. Concentrations of zeatin above optimal level (3.0 mg/L – 5.0 mg/L) also resulted in changes of plantlet morphology, such as plantlets with hyperhydricity, and stunted growth of plantlets. Hyperhydricity, also known as vitrification phenomenon is a physiological malformation associated with lack of chlorophyll, poor lignification, and over hydration of the plant tissues, leading to poor regeneration or normal mature plants [25]. High cytokinin level produced many small shoots that fail to elongate [12]. In an experiment conducted by [16], high levels of zeatin caused changes in morphology of the newly formed plant. Hence, excessive cytokinin concentration causes negative effects to growth and morphology of plantlets.

V. CONCLUSION

The results obtained suggested that cytokinins were significant growth regulator for *C. hirsuta* meristem, with opposing shooting and rooting activity. Zeanit at concentration 2.0 mg/L was the most effective cytokinin, which induced the highest shoot and leaf proliferation while minimizing root formation in nodal explants of *C. hirsuta*. Explant treated with 2.0 mg/L zeatin developed 31 shoots, and 52 leaves. Kinetin induced the highest root growth at 1.0 mg/L concentration (15 roots) therefore it was less efficient compared to zeatin. Concentrations of cytokinin above the optimal level caused toxicity to nodal explants of *C. hirsuta*, reduced growth of shoots and leaves, and in rare occasions, developed abnormal morphology.

In conclusion, in vitro regeneration can serve as an alternative propagation method for commercialization of *C. hirsuta* from nodal explants.

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