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Effect of Nitrogen Fertilizer on Yield and Amount of Alkaloids in Periwinkle and Determination of Vinblastine and Vincristine by HPLC and TLC

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Abstract: Periwinkle (*Catharanthus roseus* L.) is a most important medicinal plant which is a rich source of bioactive compounds such as alkaloids. The purpose of the present study was to investigate the effect of various concentrations of nitrogen on content and yield of alkaloids in periwinkle. This study showed that there were significant differences among various concentrations of nitrogen in all measured parameters except the fresh and dry weight of shoot tips. The results indicated that the fresh and dry weight of total foliage of periwinkle increased with increasing the amount of nitrogen fertilization from 0-100 kg N ha^{-1} and then decreased at 150 kg N ha^{-1}. The alkaloids of vinblastine and vincristine were measured by HPLC and TLC assays. The highest content and yield of vinblastine was observed in 150 kg N ha^{-1} while the lowest content and yield of vincristine was found in 150 kg N ha^{-1} by both methods of assessment. The results also showed that the content and yield of vinblastine and vincristine quantified with HPLC assay was higher than the samples determined by TLC method. The results provided important information of nitrogen effect on yield and alkaloid content of plant for commercial production of periwinkle.

Key words: Periwinkle, nutrient element, alkaloid, HPLC, TLC, Iran

INTRODUCTION

Periwinkle (*Catharanthus roseus* L.) belongs to the Apocynaceae family and is an erect handsome herbaceous perennial plant growing up to a height of 30-120 cm (Lata, 2007). This plant is a native of Madagascar which recently has been cultivated in many tropical and subtropical regions (Moreno *et al*., 1995). It is one of the important medicinal plants which has a long history of uses as antidysenteric, antiseptic, diuretic and haemorrhagic (Levy *et al*., 1983).

Popularity of periwinkle is mainly due to a protective role in cancerous, diabetic, hypertensive, dysenteric and fibrillic (Jaleel *et al*., 2006). These beneficial effects have been attributed to the high level of bioactive compounds such as alkaloids (Jaleel *et al*., 2007). The plant contains of >100 alkaloids and related compounds have so far been isolated and characterized from all parts of plant which the content of alkaloids in different parts show large variations (El-Sayed and Verpoorte, 2007). These alkaloids include vinblastine, vincristine, vincoside, catharanthine, vindoline, lochrovicine, ajmalicine, serpentine and other (Lata, 2007). The alkaloids vinblastine and vincristine present in the leaves are recognized as anticancerous drugs (Tikhomiroff and Jolicoeur, 2002). The vinblastine and vincristine were used in combination with other anticancer agents for the treatment of leukemia, lymphoma and hodgkin's diseases (Filippini *et al*., 2003).

Application of nutrient elements is a promising benefit to enhance yields of vegetative parts which produce secondary metabolites. Al-Humaid (2004) stated that the availability of essential nutrient elements necessary for datura growth and metabolism causing vigorous vegetation and high chemical production. Also, Lata (2007) reported that the fertilization of medicinal plants causes an increase in the yield of bioactive compounds. Nitrogen (N) one the most important essential nutrient elements in plants which affects all levels of plant function from metabolism to resource allocation, growth and development (Crawford, 1995; Stitt and Krapp, 1999). Previous studies have shown that application of nitrogen fertilizer causes an increase in the yield of periwinkle however, recommended dose varied considerably (Gupta, 1977; Pareek *et al*., 1985; Hegde, 1988; Rajeswara-Rao and Singh, 1990; Shylaja *et al*., 1996).

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In regard to that the high value alkaloids of vincristine and vinblastine in pharmaceutical industries, any increase in the amount of alkaloids in plant is a valuable economic aspect. Since, alkaloids are nitrogenous compounds, the availability of nitrogen is expected to play an important role in the biosynthesis and accumulation of alkaloids in plants, few published results about the effect of nitrogen application on plant alkaloids (such as vincristine and vinblastine) in the literature are available. Such data will assist in the understanding nutrient elements effect on growth and yield of plant for commercial production to meet market demand. Therefore, the purpose of the present study was to investigate the effect of various concentrations of Nitrogen (N) on yield and content of alkaloids in periwinkle and comparison two method of HPLC and TLC for screening and quantification of alkaloids of vinblastine and vincristine.

MATERIALS AND METHODS

Station and soil used for the experiment: The experiment was designed using the method of random blocks in four replications and it was performed at the Research Institute Temad in growing seasons 2008-2009. Before of deployment of the experiment, the soil (0-20 cm) was taken from upland fields were quantified physical and chemical characteristics of soil which were shown in Table 1.

Periwinkle seed: Periwinkle (*Catharanthus roseus* L.) seeds cv. G. Don were obtained from Shahid Mahlati Flower Market and screened for the uniform size. For germination inhibitors removing, the seeds were leached with distilled water for 5 days before the experiment start. Then, seeds were surface sterilized in aqueous solution of 0.1% $HgCl_2$ for 60 sec to prevent fungal attack and rinsed in several changes of sterile water. The seeds were sown in plastic pot on 22 of March and 10 weeks later, the seedlings with 5-6 leaves were transplanted in filed at a distance of 30×50 cm and irrigated immediately for better establishment.

Treatments: Nitrogen was applied at the doses of 0 (control), 50, 100 and 150 kg N ha^{-1} in the form of urea. The plants were fertilized 4 times with four equal portions of the proposed fertilizer dose in monthly intervals, starting from the day of seedling transplantation in the field. Phosphorus (40 kg P ha^{-1}) and potassium (30 kg k ha^{-1}) were applied at the time of transplantation of seedling in the field in the form of P_2O_5 and K_2O. During the experimental period, all routine agricultural practices were performed. The plants were harvested at the full bloom stage (late of September) then yield and alkaloids content were evaluated.

Total foliage and shoot tips: Fresh samples (total foliage and shoot tips) were weighted in the air on a balance of accuracy of 0.001 g. The dry matter samples (total foliage and shoot tips) were determined by drying at 70°C to a constant weight. Then, dried shoot tips were powdered by a grinder to get 40 mesh size used for vinblastine and vincristine analysis.

Determination of vinblastine and vincristine by HPLC: The HPLC system included a 600 pump, 717 Autosampler, 996 Photodiode Array Detector (PDA) and Millenium[32] Chromatography Software (all Waters, USA) and a Zorbax SBC-18 column (4.6-250 mm) (Agilent Technologies, Palo Alto CA). The mobile phase composition was 5 mM potassium dihydrogenphosphate solution (pH adjusted to 6 with H_3PO_5) and acetonitrile (85:15, v/v). The flow rate was 1 mL min^{-1} and 10 μL portions were injected into the column. Detection was performed at a wavelength of 297 nm under a constant temperature (35±1°C). The retention time and UV spectrum of the peak compared to the standard were used to identify the compound and to check the purity. Quantitative determination was carried out using calibration graphs obtained from standard solutions of vinblastine and vincristine diluted in methanol at a concentration range of 0.1-100 mg mL^{-1}.

The vinblastine and vincristine were estimated with High Performance Liquid Chromatography (HPLC) according to the method described by Raper *et al*. (1978). Briefly, approximately 3 g of dry shoot tips were homogenized in methanol (30 mL) and then the homogenate was refluxed for 6 h. After refluxing, the refluxed sample was filtered in vacuo. The filtrate was evaporated to dryness and then 10 mL water and HCl and 10 mL chloroform was added. The eluted was collected in a flask and then evaporated to dryness under reduced pressure below 40°C using a rotary evaporator. The residue was dissolved in 2 mL of methanol and the sample solution was filtered through a 0.5 μm filter before injection into the HPLC system. The results were expressed as g m^{-2} for vinblastine and vincristine yield and as % for vinblastine and vincristine content.

Determination of vinblastine and vincristine by TLC: The vinblastine and vincristine were measured after separation

Table 1: Some physical and chemical analysis of soil

Depth (cm)	Texture	pH	EC (ds m^{-1})	Organic C (%)	Total N (%)	Available P (ppm)	Available K (ppm)	Fe (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-})	Mn (mg kg^{-1})
0-20	Sand-loam	7.21	4.12	0.43	0.15	13.28	335	7.32	1.57	1.04	7.31

of methanol extracted alkaloids with Thin Layer Chromatography (TLC) according to the method of Monforte-Gonzalez. The separated TLC bands were identified by dragendorf reagent. Bands were scrapped from TLC plate, dissolved in methanol and analyzed spectrophotometerically for vinblastine and vincristine. The results were expressed as g m^{-2} for vinblastine and vincristine yield and as % for vinblastine and vincristine content.

Statistical analysis: Data were analyzed by Statistical Analysis System (SAS) software Version 9.1 Using Analysis Of Variance (ANOVA) and differences among means were determined for significance at $p<0.05$ using Tukey's test.

RESULTS AND DISCUSSION

Total foliage and shoot tips: The effect of nitrogen fertilizer doses on total foliage and shoot tips of periwinkle analyzed was shown in Fig. 1 and 2. The results show that dose variations of nitrogen had significant influence on the fresh and dry weight of total foliage (Fig. 1) but no significant influence on the fresh and dry weight of periwinkle shoot tips (Fig. 2).

The fresh and dry weight of total foliage and shoot tips increased with the increase of nitrogen doses from 0-100 kg N ha^{-1} but decreased at 150 kg N ha^{-1} (Fig. 1 and 2). The results were in agreement with the findings reported by Sreevalli *et al.* (2004). Similar results also reported for other plants such as spinach (Elia *et al.*, 1999) salvia, radish (Liao *et al.*, 2009) and onion (Biesiada and Kolota, 2009). Thus, one can say that there is a positive relation between plant yield and nitrogen fertilizer.

The results showed that application of higher rates (150 kg N ha^{-1}) of nitrogen fertilizer causes a decrease in the yield of periwinkle. Rajeswara-Rao and Singh (1990) reported that the application of higher doses of nitrogen resulted in a lower effectiveness of nitrogen absorption. According to Al-Humaid (2004) the excess amount of fertilizers caused burning and death of the root hairs affecting negatively the root growth by inhibiting the elongation and enlargement of the root cells, consequently limited the extension of the roots in the soil as they became weak, short and fluffy. Consequently, the burning and death of the root hairs resulting from high nitrogen fertilizer supply led to a lower effectiveness of nitrogen absorption that causes a decrease in the yield of periwinkle. Results recorded in this study confirm these findings since, the highest dose of nitrogen fertilization caused substantial reductions in the fresh weight and dry weight of shoot tips and total foliage of periwinkle.

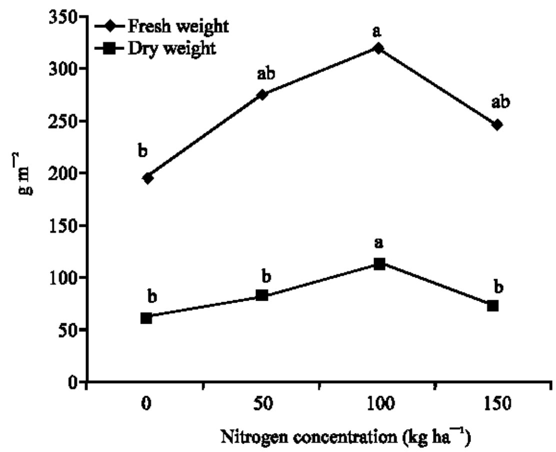

Fig. 1: Effect of nitrogen fertilization on fresh and dry weight of total foliage of periwinkle, points marked with the different letters are significantly different ($p<0.05$)

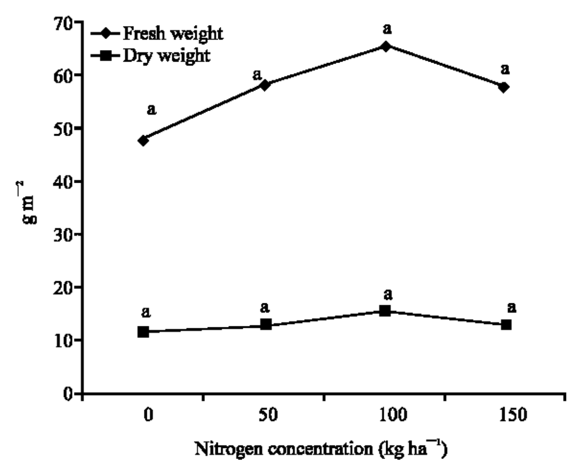

Fig. 2: Effect of nitrogen fertilization on fresh and dry weight of shoot tips of periwinkle, points marked with the different letters are significantly different ($p<0.05$)

Vinblastine and vincristine: The results influences of various nitrogen doses on the vinblastine content and yield were determined by HPLC and TLC assays are shown in Fig. 3 and 4. Nitrogen treatments had significant effects on vinblastine content and yield of periwinkle.

The vinblastine concentration increased linearly by increasing nitrogen rate of application and reached a maximum values at 150 kg N ha^{-1}. The vinblastine content at four different of nitrogen doses measured by HPLC assay comprised 0.011, 0.012, 0.015 and 0.018% while by TLC assay comprised 0.002, 0.0024, 0.0028 and 0.0048%, respectively (Fig. 3). The results of HPLC and TLC analysis also showed that the vinblastine yield increased with the increase of nitrogen doses from 0-150 kg N ha^{-1}

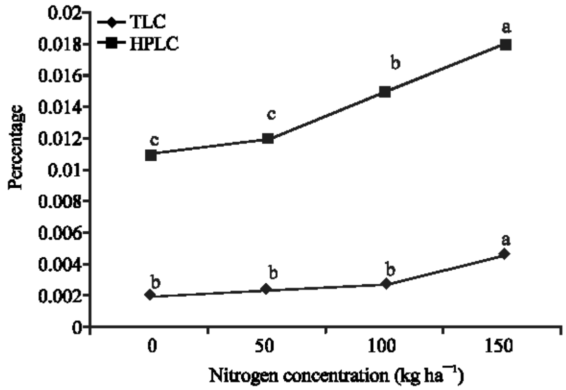

Fig. 3: Effect of nitrogen fertilization on vinblastine content of periwinkle (determination of vinblastine by HPLC and TLC methods), points marked with the different letters are significantly different ($p<0.05$)

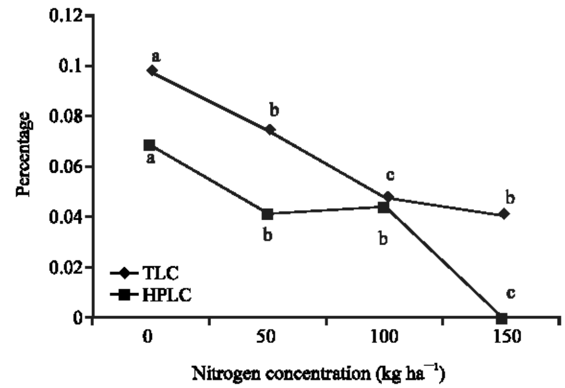

Fig. 5: Effect of nitrogen fertilization on vincristine content of periwinkle (determination of vincristine by HPLC and TLC methods), points marked with the different letters are significantly different ($p<0.05$)

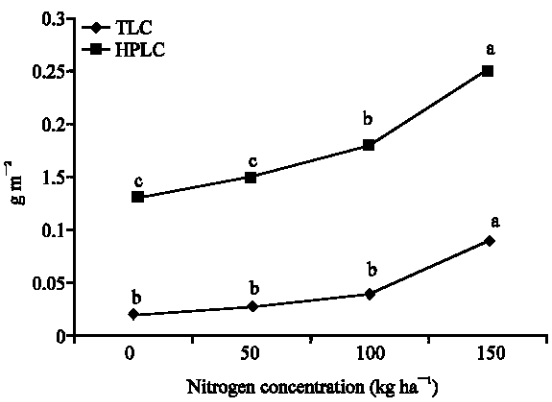

Fig. 4: Effect of nitrogen fertilization on vinblastine yield of periwinkle (determination of vinblastine by HPLC and TLC methods), points marked with the different letters are significantly different ($p<0.05$)

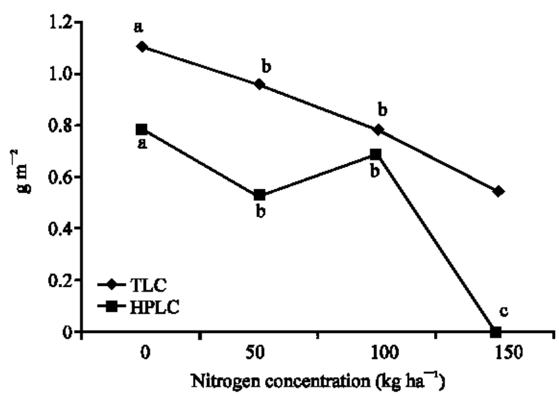

Fig. 6: Effect of nitrogen fertilization on vincristine yield of periwinkle (determination of vincristine by HPLC and TLC methods), points marked with the different letters are significantly different ($p<0.05$)

(Fig. 4). Abdolzadeh *et al.* (2006) have been reported that the content vinblastine of periwinkle plant increased with increasing nitrogen doses form 2.75-11 mM in the medium.

The various levels effect of nitrogen on the vincristine content and yield were determined HPLC and TLC assays are shown in Fig. 5 and 6. The results showed that various doses of nitrogen had significant effect on the vincristine content and yield of periwinkle.

In the present study, the vincristine content decreased linearly with the increasing nitrogen dose and the lowest vincristine content determined by HPLC and TLC was observed with a nitrogen dose equal to 150 kg N ha^{-1} (Fig. 5). The yield of vincristine decreased linearly by increasing nitrogen levels of application, reaching a minimum of 0.54 mg m^{-1} (HPLC) and 0 mg m^{-1} (TLC) at 150 kg N ha^{-1} (Fig. 6).

Nitrogen is a constituent of the alkaloids which play an important role in the synthesis of alkaloids. Therefore, increasing nitrogen nutrient leads to an increase in alkaloid content. Also, it assumed that this increment of alkaloid content was usually attributed to the increase of herb yield due to fertilization. Lata and Sadowska (1996) reported that the concentration and yield of total alkaloid increased linearly by increasing nitrogen rate of application in periwinkle. Also, Al-Humaid (2004) showed that nitrogen fertilization causes an increase in alkaloid content in datura plant at moderate fertilizer doses while at high doses, a decrease was observed.

Not enough research is available regarding the effect of nitrogen fertilizer on vinblastine and vincristine (content and yield) of periwinkle or other medicinal plant. The results of the study showed that the content and yield of vinblastine increased significantly while the vincristine content and yield decreased significantly by increasing nitrogen concentration of fertilization in periwinkle.

Comparison HPLC and TLC assays: According to results, the content and yield of vinblastine and vincristine quantified with the HPLC assay was higher than the samples determined by the TLC method (Fig. 3-6). The High Performance Liquid Chromatography (HPLC) analysis showed good precision and a notably lower limit of detection with good reproducibility as compared to the Thin Layer Chromatography (TLC) technique. This observation does not void the fact that the TLC technique is remarkably suitable for mass screening and routine analysis which can be used when HPLC is not available.

CONCLUSION

Statistically significant differences were observed between various concentrations of nitrogen in all measured factors except shoot tips fresh and dry weight. The data indicated that the content and yield of vinblastine increased while the content and yield of vincristine decreased with increasing the amount of nitrogen fertilization from 0-150 kg N ha^{-1} by both methods of assessment. In addition, the results provide important information on nitrogen effect on yield and alkaloid content of plant for commercial production of periwinkle. The assay of HPLC has most efficient for screening and quantification of vinblastine and vinblastine than the TLC assay. Periwinkle is a rich source of alkaloids can be classified as a high nitrogen demanding plant but obviously for obtain a high of yield and bioactive compounds content are needed to other of nutrient elements.

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