Journal of Medicinal Materials, 2022, Vol. 27, No. 6 (pp. 373 - 378)

CONFIRMATION OF THE PRESENCE OF *DRYNARIA SPARSISORA* (DESV.) T. MOORE IN VIETNAM: EVIDENCE BASED ON MORPHOLOGICAL AND MOLECULAR DATA

Nguyen Thi Lan Hoa*, Nguyen Khuong Duy*, Lai Viet Hung, Pham Thanh Huyen, Tran Duc Trung, Nguyen Hoang, Phan Van Truong, Nguyen Quynh Nga, Dang Minh Tu, Nham Minh Phuc, Le Thi Tu Linh

Medicinal Material Resources Center, National Institute of Medicinal Materials

*Corresponding author: lanhoanimm@gmail.com

(Received November 10th, 2022)

Summary

Confirmation of the Presence of *Drynaria sparsisora* (Desv.) T. Moore in Vietnam: Evidence Based on Morphological and Molecular Data

The studied *Drynaria* specimen was collected in Phu Quoc National Park in 2022. A combination of morphological assessment of scale and rhizome characteristics and molecular analysis of the plastid *rbcL* supported that the collected specimen is *D. sparsisora.* Thus, the species is confirmed in Vietnam. According to our preliminary survey, the species can be found scattered in several small populations on Phu Quoc Island. Further investigations into broader aspects of this species, i.e., natural habitat and distribution as well as its biological, cytological characteristics, phytochemistry, and medicinal usage should be considered to facilitate proper protection and sustainable promotion as a new herbal material.

Keywords: *Drynaria sparsisora, Taxonomy, rbcL, Phu Quoc, Vietnam.*

1. Background

The fern genus *Drynaria* (Bory) J.Sm is native to an extensive geographic range from the Tropical and Subtropical Old World to the Pacific region. Commonly known as epiphytes in tropical forests, the genus includes about 33 accepted species that have certain ornamental and medicinal values [1]. The greatest species diversity of *Drynaria* is found in continental Asia. The phylogenetic classification of drynaroid ferns is still ambiguous. Both humus-collecting leaves (fronds) and plastid fragments-based approaches revealed that *Drynaria* is paraphyletic with regard to *Aglaomorpha* [2]. However, the proposal of combining these two genera possessing few separatable morphological characters is not yet officially accepted [1]. Nonetheless, for the well-established *Drynaria* genus itself, the morphology of fronds and sporangium are key characteristics for species discrimination studies.

In Vietnam, seven species have recently been recorded for the genus *Drynaria* with different geographic distributions and richness. Those include *D. bonii* scatteringly found throughout the country, *D. fortunei* and *D. propinqua* found in Northern mountainous areas, *D. quercifolia* found in Southern areas, and *D. parishii*, *D. rigiduala* recorded in the Central Highland. Five of those seven *Drynaria* recorded species have been commonly known for their medicinal values, especially two species *D. fortunei* and *D. bonii* have been listed in the Vietnam Red Book whereas *D. delavayi* has not yet been practically recorded [3].

In this study, a specimen of the eighth *Drynaria* species in Vietnam, *D. sparsisora,* was examined and confirmed employing both morphological and molecular measures. This result provides significant supplementary information on the new fern species for the Phu Quoc National Park.

2. Materials and methods

Examined specimens

Vietnam. Phu Quoc Island. Apo Range 10°21'53.34"N; 103°59'45.27"E, 156 m asl 21 April 2022, voucher: LVH-D19, specimen NIMM0019249 (Table 1).

Morphological observations

Specimens of *D. sparsisora* and *D. quercifolia* collected in Vietnam and elsewhere as references (PE, E, HN, K, MO, P, US) were examined.

Plant morphology was observed and photographed with a camera (Canon EOS 70D);

stereomicroscope (Zeiss Stemi 2000-C equipped with a Zeiss Axiocam ERC 5s digital camera) and microscope (Olympus BX53 equipped with an Olympus SC180 camera). Terminology for leaves, rhizome, and other characterizations was done as described by [4] and following [5].

Table 1. Information of 6 *Drynaria* specimens sampled across Vietnam representing *D. sparsisora* and *D. quercifolia*. All specimens were deposited in the herbarium of the National Institute of Medicinal Material.

No.	Voucher	Taxonomy treatment	Herbarium ID	Location	Collector
1.	NLH-D13	*Drynaria quercifolia*	NIMM0019221	Sa Thay, Kon Tum	N.L. Hoa
2.	LVH-D14	*Drynaria quercifolia*	NIMM0019244	Nui Ba Den, Tay Ninh	L.V. Hung
3.	LVH-D19	*Drynaria sparsisora*	NIMM0019249	Phu Quoc, Kien Giang	L.V. Hung
4.	PTH-D25	*Drynaria quercifolia*	NIMM0019250	Vinh Cuu, Dong Nai	P.T. Huyen
5.	LVH-D26	*Drynaria quercifolia*	NIMM0019267	Vinh Cuu, Dong Nai	L.V. Hung
6.	LVH-D27	*Drynaria quercifolia*	NIMM0019268	Vinh Cuu, Dong Nai	L.V. Hung

Molecular data and phylogenetic analysis

Six specimens representing two morphologically recognized species *D. sparsisora* and *D. quercifolia* sampled across Vietnam were used for molecular analysis (Table 1). Total genomic DNA was extracted from silica-dried leaves using the DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions. The integrity of gDNA samples was assessed by 1% agarose gel electrophoresis while the concentration and purity were determined using a Quickdrop Spectrophotometer (Molecular Devices, USA). A primer pair specific for *Drynaria*'s chloroplast *rbcL* region were designed based on published records deposited in GenBank (*rbcLdF*: 5'GCTGAAACGGGGGAAATTAAA-3' and *rbcLdR*: 5'CGTCTTACAACTATTGCGGAG-3') for amplification of ca. 800bp fragment from chloroplast *rbc*L gene. The PCR cocktails containing 12.5µl Phusion High-Fidelity 2X PCR Master Mix (Thermo Scientific, USA), 0.5µM of each primer, and 25-50 ng of genomic DNA were prepared in a total 25µl reaction. The amplifications were performed on a Mastercycler x50s (Eppendorf, Germany) using the following program: 30s at 98°C for initial denaturation, 35 amplification cycles (each consisting of 30s at 98°C for denaturation, 30s at 55°C for annealing, and 30s at 72°C for extension), 2 min at 72°C for extension, and a final hold at 16°C. The amplified products were purified using PCR Purification Kit (Qiagen, Germany) as described by the manufacturer, then validated on 1% agarose gel for amplification specificity before being sequenced using Applied Biosystems™ 3500 XL Genetic Analyzer.

Raw sequencing data were checked and trimmed for the qualified *rbcL* region using Geneious v11.1.5. A nucleotide matrix assembled from six newly generated sequences obtained from collected *Drynaria* specimens, 27 reference *rbcL* regions extracted from full chloroplast genomes of 15 *Drynaria* species, and that from *Loxogramme lankokiensis* as an outgroup according to PPG I [6] was locally aligned using MUSCLE v.3.8.425.

Species identification was done employing comparative tree-based analysis to evaluate whether species were recovered as monophyletic with the established *rbcL* sequence matrix. The aligned matrix was subjected to two approaches i.e., a maximum likelihood (ML) method using PhyML 3.0 package [7] and a neighbor-joining (NJ) method using MEGA v.11.0.13 [8]. The best fit ML nucleotide substitution models for each approach were both estimated as K80/K2 + G using Smart Model Selection (SMS) tool [9] and built-in model selection module in MEGA v.11.0.3 software, respectively. The bootstrap analysis of 1,000 replicates was applied to both approaches. The generated ML and NJ phylogenetic trees were visualized and annotated using iTOL v6 tool (https://itol.embl.de/). The discriminated species with multiple accessions resolving as a monophyletic clade was recorded using the bootstrap support (BS) > 70%.

All described works were conducted at the Medicinal Material Resources Center, National Institute of Medicinal Materials from January 2022 to November 2022.

3. Results and discussion

Morphological description of D. sparsisora

Description: Rhizome short, thick, and creeping, 1.0 - 3.0 cm in diam., fleshy, densely scaly, smooth, and snake-like in shape; covered with dark scales, the narrow apical portions having broken off at the old part; scales peltate, appressed, overlapping, narrowed abruptly above the rounded base, tapering to a narrow, apex acute, 1-6 mm × 1-2 mm, stiff, brown to very dark brown, margins paler and occurring a layer of hair-like. A dark brown, prominent midrib was observed on the scales. Basal fronds ovate, smooth, overlapping, 15-30 cm x 10-20 cm, margins deeply lobed, and lobed-apex rounded. Foliage fronds stiffly erect, petioles 3-12 cm long, leathery lamina with long winged petioles, 20-50 cm x 15-20 cm, 7-11 pair deep lobes, the lobes strap-like, narrowed slightly towards the base, tapering to a mostly acute apex, veins clear on the surface; veins of base fronds were reticulate between two main lateral veins; free veinlets were simple or absent. Sporangia round, arranged in two or more irregular rows between lateral veins of the foliage leaves; the spores are nearly round or rounded slightly sunken, and covered with a layer of spiny hairs (Fig. 1A-D).

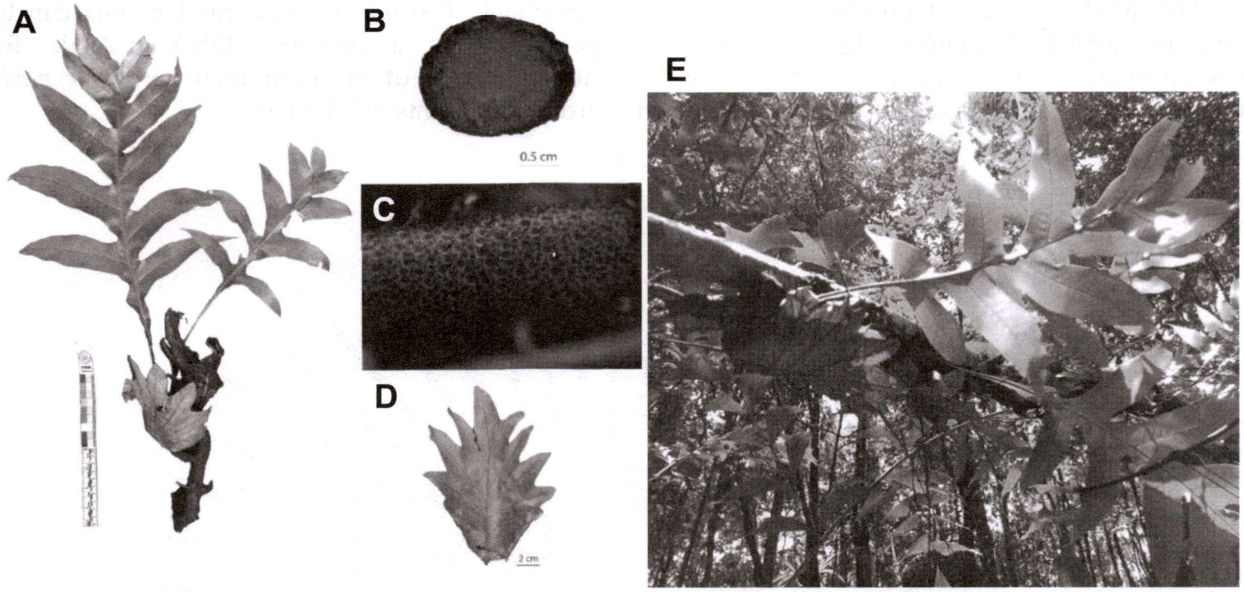


Fig. 1. Morphological observations of *Drynaria sparsisora* specimen collected from the Phu Quoc National Park. (A) Lifeform; (B) Slice of rhizome; (C) Rhizome scales; and (D) Basal frond; (E) Epiphyticaly grew *Drynaria sparsisora* on the tree trunks in the core of the Phu Quoc National Park.

Habitat and distribution: Epiphytic, epilithic, or terrestrial fern on tree trunks in the tropical moist evergreen rainforest, at about 156 m asl (above sea level) in Vietnam (the Phu Quoc National Park, Phu Quoc Island, Kien Giang) (Fig. 1E). *Drynaria sparsisora* was also recorded in the tropical lower mountainous rain forest amongst leaf litter, at about 900 m asl in Thailand, and about 1,100 m asl in the Philippines and was observed on a rock crevice close to a stream bank of the lower Hantane area in Srilanka [10]. It was also reported that *D. sparsisora* was found occasionally in sandy soils or on rocks; it tolerates more exposure than *D. quercifolia* [11].

Phylogenetic analysis and species discrimination based on plastid rbcL sequence

Using the newly designed specific primer pair, the *rbcL* regions were successfully recovered from all analyzed specimens. In total, 38 chloroplast *rbcL* sequences (up to 640bp) of 16 *Drynaria* species, including 28 reference sequences derived from GenBank-deposited complete chloroplast genomes, and an outgroup species were compiled for phylogenetic analysis. As the trees obtained from the ML and NJ analyses were almost identical in their topologies with strong bootstrap support (BS) values and some different placements of the same accessions observed within some monophyletic clades, only

the ML tree based on the plastid *rbcL* matrix is presented (Fig. 2).

The ML tree contains seven confident monophyletic clades corresponding to seven *Drynaria* species/species complexes with high conformity in phylogenetic placements of sister species as revealed in *Drynaria* relationship treatment based on complete chloroplast genomes i.e., *D. bonii* and *D. quercifolia*, *D. sinica* and *D. delavayi* [12]. The LVH-D19 accession was confidently resolved as of *D. sparsisora* species since its *rbcL* sequence and those of the other two references (AB248095 and AY529167) settled a strongly supported monophyletic clade (BS = 84.43%). The *D. sparsisora* clade is sister to a group combining clades of two closed relatives *D. bonii* and *D. quercifolia* (BS = 95%).

The phylogenetic relationship of *Drynaria* and its affiliated genera *Aglaomorpha* and *Christiopteris* is difficult to resolve unambiguously due to the discrepancy between the morphological approach based on complex morphological patterns and the molecular approach utilizing plastid fragments [1],[2]. Nevertheless, humus-collecting leaves and sporangium arrangement are still specific morphological measures for effective interspecific discrimination of *Drynaria* members. The increase in the amount of plastid sequence data, especially those of complete chloroplast sequences has provided robust evidence elucidating the phylogenetic relationship of *Drynaria* and related species [12]. In this study, the reconstructed ML phylogenetic tree of *Drynaria/ Aglaomorpha* species solely based on the plastid *rbcL* region successfully resolved the placement of all examined accessions in accordance with their taxonomy treatment. The plastid *rbcL* marker thus can be promoted as a potential DNA barcode for authentication of the traditional herbs *Drynaria* from adulterants in Vietnam.

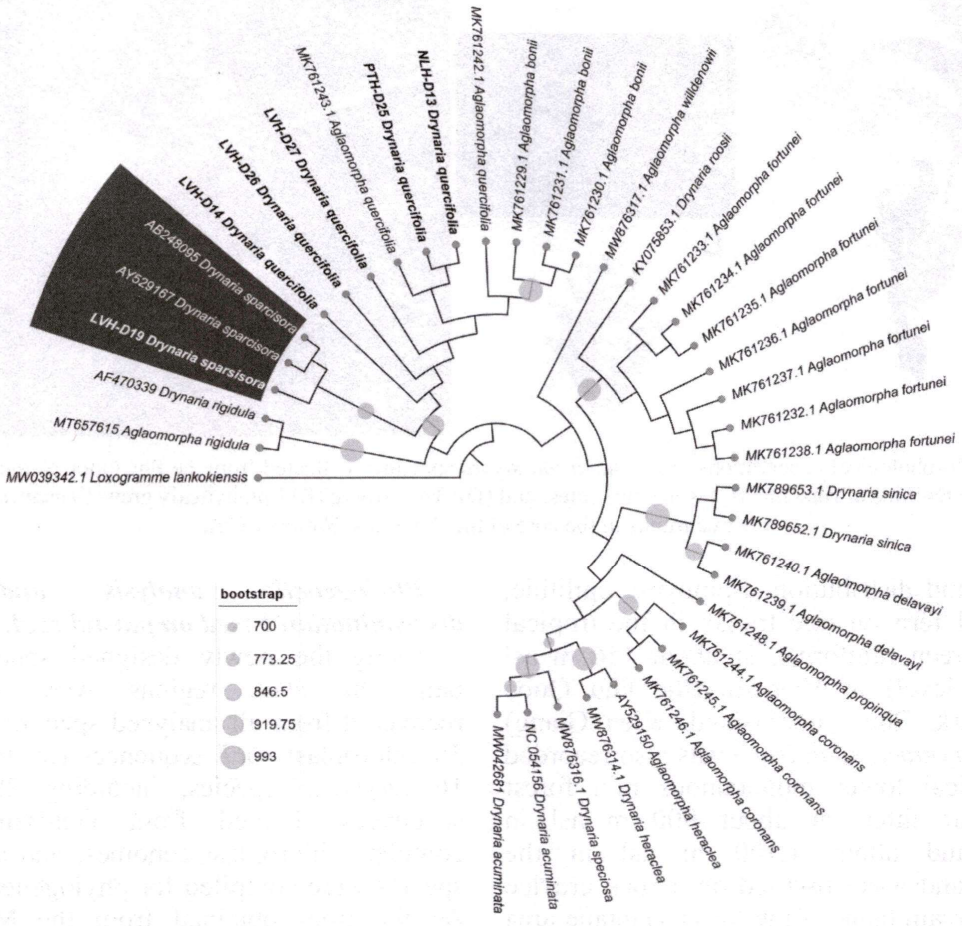
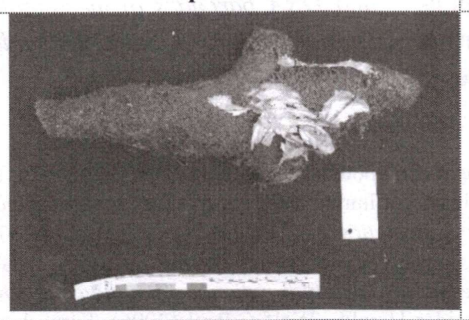
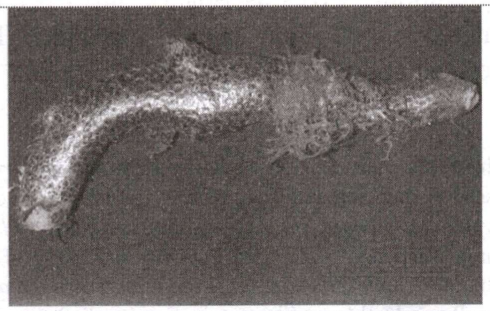
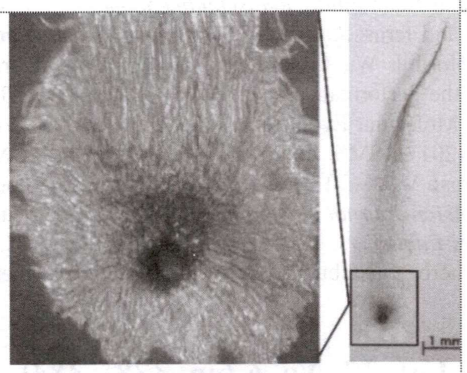
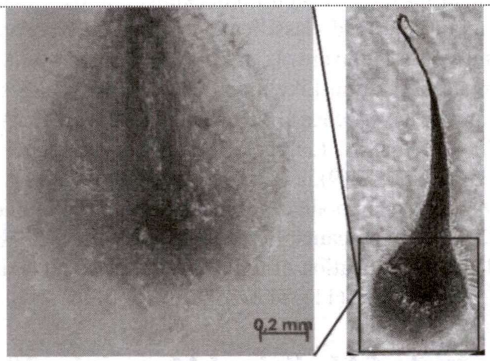
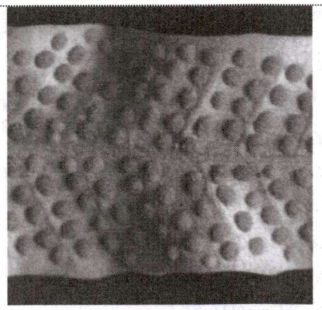
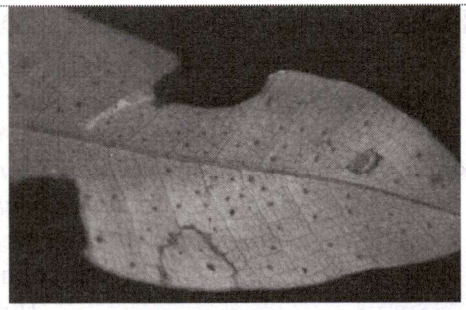
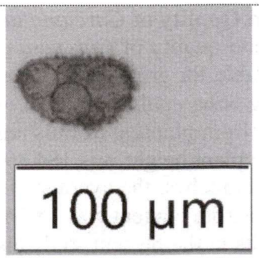
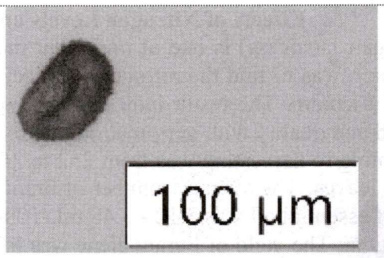


Fig. 3. ML phylogenetic positions based on plastid *rbcL* marker of six *Drynaria* accessions collected across Vietnam (in bold) in the correlation with 27 reference sequences representing 15 *Drynaria* species. The bootstrap support values (cutoff 700/1,000 = 70%) were visualized accordingly. The gray-shaded clade indicates evidence of the first recorded *D. sparsisora* in Vietnam. The *D. sparsisora* clade is sister to a group combining clades of two closed relatives *D. bonii* and *D. quercifolia*

Comparative morphological characterization of D. sparsisora and D. quercifolia

In general, *D. sparsisora* resembles *D. quercifolia* which is one of the most common epiphytic ferns. Without rhizomes and sori, it is almost impossible to practically distinguish these two species. In addition, *D. quercifolia* can be distinguished by its wooly, larger rhizome; feather-like scales, which are usually lighter in color, have an oval-shaped base, and has no prominent midrib and two regular rows of sori (Table 2).

Table 2. Comparative morphological characterization of *D. sparsisora* and *D. quercifolia*

	D. quercifolia	**D. sparsisora**
Rhizome		
	Large wooly, fibrous, lizard-like shape	Short, thick and fleshy creeping rhizome, snake-like in matured
Scale		
	Lighter color, feather-like scales, ovale-shaped base, and no prominent midrib	Dark brown scale, broken off narrow apical portions; rounded base, prominent midrib
Sporangium arrangement		
	Two regular rows of sori	Two irregular rows of sori
Spore (mag. X40)		
	Similar: rounded sunken spore with spikes	

4. Conclusion

Scale and rhizome characters with information on the previously described specimens suggested that the LVH-D19 sample collected in Phu Quoc National Park is of *Drynaria sparsisora*. This treatment was strongly supported by molecular evidence based on the plastid *rbcL* marker. According to our preliminary survey, *D. sparsisora* only naturally exists as isolated small populations scattered on Phu Quoc Island. Further investigations are thus required to gain a deeper insight into biological and cytological characteristics as well as the phytochemistry profile and medicinal potential usage of this newly recorded species for proper protection and sustainable promotion in traditional medicine.

Acknowledgments: *This study was supported by the National Institute of Medicinal Materials (project: Establishing data on morphological characteristics and DNA barcodes of medicinal plants belonging to Drynaria (Bory) J. Sm. and its adulterate).*

References

1.Christenhusz M. (2015), New combinations in *Drynaria* (Polypodiaceae subfam. Polypodioideae), *Phytotaxa*, 230, 299. **2.** Janssen T. and Schneider H. (2005), Exploring the evolution of humus collecting leaves in drynarioid ferns (Polypodiaceae, Polypodiidae) based on phylogenetic evidence, *Plant Systematics and Evolution*, 252(3), 175-197. **3.** Luong Thi Hong Nhung, Ha Minh Tam, Do Thi Xuyen (2013), The morphology of genus-*Drynaria* (Bory) J. SM. (Polypodiaceae) in Vietnam, *Scientific report on ecology and biological resources (The fifth national scientific conference)*, 203-208. **4.** Smith A.R., Pryer Kathleen, Shuettpelz E., Korall Petra, Scheider Harald., Wolf G.Paul (2006), Fern classification, *Biology and Evolution of Ferns and Lycophytes,* 159-174. **5.** Desvaux A. N. (1811), Der Gesellschaft Naturforschender Freunde zu Berlin Magazin für die neuesten Entdeckungen in der gesammten Naturkunde, *Realschulbuchhandlung*, 5, 315. **6.** PPG I (2016), A community-derived classification for extant lycophytes and ferns, *Journal of Systematics and Evolution*, 54(6), 563-603. **7.** Guindon S., Dufayard J., Lefort V., Anisimova M., Hordijk W., Gascuel O. (2010), New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0, *Systematic Biology*, 59(3), 307-321. **8.** Tamura K., Stecher G., Kumar S. (2021), MEGA11: Molecular Evolutionary Genetics Analysis Version 11, 38(7), 3022-3027. **9.** Lefort V., Longueville J.-E. , and Gascuel O. (2017), SMS: Smart Model Selection in PhyML, *Molecular Biology and Evolution*, 34(9), 2422-2424. **10.** Lubos L.C., Amoroso V. B. (2011), The Identity and Morphology of the Three Species of *Drynaria* (Polypodiaceae) in Bukidnon, Philippines, *Asian Journal of Biodiversity*, 2(1). **11.** de Winter W. P., Amoroso V. B. (2003), Cryptogams: Ferns and Fern Allies, *Backhuys Publishers*. **12.** Shen Z., Lu T., Zhang Z., Cai C., Yang J., Tian B. (2019), Authentication of traditional Chinese medicinal herb "Gusuibu" by DNA-based molecular methods. *Industrial Crops and Products*, 141(11), 111756.

Journal of Medicinal Materials, 2022, Vol. 27, No. 6 (pp. 378 - 384)

EFFECTS OF NITROGEN LEVELS ON GROWTH, YIELD AND QUALITY OF *CURCUMA AERUGINOSA* ROXB.

Nguyen Ba Hung[1], Dao Van Nui[1,*], Dang Thi Ha[1], Tran Dai Hai[1], Trinh Van Vuong[1], Phan Thi Thu[1], Nguyen Van Tai[2], Phung Nhu Hoa[2]

[1]Hanoi Research Centrer for Cultivation and Processing of Medicinal Plants, National Institute of Medicinal Materials (NIMM), Hanoi, Vietnam;

[2]National Institute of Medicinal Materials (NIMM), Hanoi, Vietnam

*Corresponding author: vannui102@gmail.com

(Received July 30th, 2022)

Summary

Effects of Nitrogen Levels on Growth, Yield and Quality of *Curcuma aeruginosa* Roxb.

Nitrogen factor (N) is one of important major effect to yield and quality of *Curcuma aeruginosa* Roxb. The purpose of this research was to find the nitrogen fertilizer formula that is suitable for growth and development of *Curcuma aeruginosa* Roxb. in Vietnam. The result indicated that application of 150 kg N/ha could help *Curcuma aeruginosa* Roxb. to achieve high yield and quality with germination rate reached 80%, the time from planting to finishing sprouting was 20-25 days, time from planting to harvesting was from 252 to 260 days; The average plant height was 184.89 cm; number of leaves/main stem was 9-10 leaves; The average number of branches/cluster was 3-4 branches; the average yield was 8.87 - 10.37 tons/ha. The content of essential oil was 3.12 - 3.48 mL/100g; the yield of Germacrom content was 15.364 kg/ha; the yield of Curdion was 53.494 kg/ha; The yield of Furanodiene was 8.756 kg/ha. The results in the present study also could enable the production of *Curcuma aeruginosa* Roxb. obtain the high quality medicinal material in the future.

Keywords: *Curcuma aeruginosa* Roxb., *Nitrogen dosage on growth, Yield, Essential oil.*