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Limited distribution of natural cyanamide in higher plants: Occurrence in *Vicia villosa* subsp. *varia*, *V. cracca*, and *Robinia pseudo-acacia*

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Abstract

Cyanamide (NH_2CN) has recently been proven to be a natural product, although it has been synthesized for over 100 years for agricultural and industrial purposes. The distribution of natural cyanamide appears to be limited, as indicated by our previous investigation of 101 weed species. In the present study, to investigate the distribution of natural cyanamide in *Vicia* species, we monitored the cyanamide contents in *V. villosa* subsp. *varia*, *V. cracca*, and *V. amoena* during their pre-flowering and flowering seasons. It was confirmed that *V. cracca* was superior to *V. villosa* subsp. *varia* in accumulating natural cyanamide, and that *V. amoena* was unable to biosynthesize this compound under laboratory condition examined. The localization of cyanamide in the leaves of *V. villosa* subsp. *varia* seedlings was also clarified. In a screening study to find cyanamide-biosynthesizing plants, only *Robinia pseudo-acacia* was found to contain cyanamide among 452 species of higher plants. We have investigated 553 species to date, but have so far found the ability to biosynthesize cyanamide in only three species, *V. villosa* subsp. *varia*, *V. cracca* and *R. pseudo-acacia*.

Keywords: *Vicia villosa* subsp. *varia*; *Vicia cracca*; *Vicia amoena*; *Robinia pseudo-acacia*; Leguminosae; Distribution; GC–MS; Cyanamide

1. Introduction

Despite the previously common understanding that cyanamide (NH_2CN) is only a synthetic compound, its natural occurrence has recently been confirmed (Kamo et al., 2003). The oldest synthetic fertilizer is a hydrolyzed product of calcium cyanamide, which was first synthesized in 1898: the formation of cyanamide is rapid when in contact with water molecules in soils. The isolation and characterization of a cyanamide hydratase from a soil fungus demonstrated a high specificity for cyanamide in its hydration to urea. This was unexpected because a clear interpretation could not be given as to why the soil fungus has adapted to such a recently introduced substance (Maier-Greiner et al., 1991; Estermaier et al., 1992). However, cyanamide has been isolated from *Vicia villosa* Roth subsp. *varia* (Host.) Corb. as a plant-growth-inhibitory compound (Kamo et al., 2003). Its natural origin has been proven by demonstrating the incorporation of ^{15}N-labelled nitrate into cyanamide using a seedling of this leguminous plant (Kamo et al., 2006a). To quantify the cyanamide content in plants with high accuracy, we have developed a stable isotope dilution gas chromatography–mass spectrometry (SID-GC–MS) method, in which an extract from a plant is purified after adding an internal standard, $[^{15}N_2]$-cyanamide, and analyzed using GC–MS (Hiradate et al., 2005). Using this method, we have analyzed 101 species

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of common weeds in Japan and found that only *V. cracca* L. and *V. villosa* subsp. *varia* contained cyanamide among them (Kamo et al., 2006b). The cyanamide content in the former was about ten times larger than in the latter, although analysis was conducted for each single plant material collected at one location at one growth stage.

Here, we show changes of the cyanamide contents in the wild aerial parts of *V. cracca*, *V. villosa* subsp. *varia*, and *V. amoena* Fisch. throughout their pre-flowering and flowering seasons. To avoid ambiguity which might be derived from environmental factors, we also analyzed the cyanamide contents in seedlings of the three species cultivated in a growth chamber. Additionally, we conducted an extensive survey on 452 species of higher plants in order to clarify the distribution of cyanamide-biosynthesizing species.

2. Results and discussion

We monitored the changes of the cyanamide contents in the aerial parts of *V. villosa* subsp. *varia*, *V. cracca*, and *V. amoena* grown in the wild. While in our previous study we found the presence of cyanamide in *V. villosa* subsp. *varia* (mentioned without "subsp. *varia*" in our previous report) and *V. cracca* and its absence in *V. amoena* (Kamo et al., 2006b), the morphologies of the three species closely resembled each other: the one-sided racemes consist of cascading blue to pink flowers emerging from the leaf axilla, and the leaves are pinnate with pairs of leaflets 5–10 mm in length. To confirm the difference of cyanamide contents, analyses of multiple samplings over a long-term period needed to be conducted. We thus sampled the aerial parts of *V. villosa* subsp. *varia* at three locations between May to July, 2006. The cyanamide content was about 500 mg/kg fr. wt on June, when the flowers were in full bloom (Fig. 1). The foliage rapidly withers and dies after flowering, which is characteristic of this species. *Vicia cracca* flowers between June to October, and the full-blooming season at the sampling site is August. The value of the cyanamide content reached nearly 1500 mg/kg fr. wt. For *V. amoena*, we continued sampling between July and September and found that all the samples tested contained no detectable cyanamide (<1 mg/kg fr. wt). In our previous paper, we reported that the cyanamide contents of *V. villosa* subsp. *varia*, *V. cracca* and *V. amoena* were 449 ± 70, 3,526 ± 61, and <1 mg/kg fr. wt, respectively, on a single sampling (Kamo et al., 2006b), corresponding to those of the present study. It is likely that the cyanamide accumulation is greater in *V. cracca* than in *V. villosa* subsp. *varia* and that of *V. amoena* is negligible. It is inappropriate, however, to conclude that *V. cracca* is superior to *V. villosa* subsp. *varia* in cyanamide biosynthesis from the field experiment because of the large standard deviations shown in Fig. 1, probably derived from the different growth conditions.

To avoid the effects of uneven environmental factors in the field, such as temperature, light intensity, and nutrient and water conditions on the cyanamide content of plants, we quantified cyanamide in seedlings cultivated in an illuminated growth chamber. By cultivating each species under identical conditions, it became possible to compare the character intrinsic to each species regarding cyanamide biosynthesis. Seeds of the three *Vicia* species planted in vermiculite were grown without fertilizer for ten weeks. After absorbing water, the seeds of *V. villosa* subsp. *varia* began germinating without any treatment, while those of *V. cracca* and *V. amoena* needed injury- or sulfuric acid-treatments to elicit high germination (Table 1). Under identical conditions, *V. villosa* subsp. *varia* significantly showed the fastest growth among the three. This may be because the species has been bred for commercial use in pasture. The cyanamide contents in the seeds of the three species were less than the detectable level (<1 mg/kg fr. wt), indicating that virtually no cyanamide is carried over to the next generation and that its biosynthesis is activated after

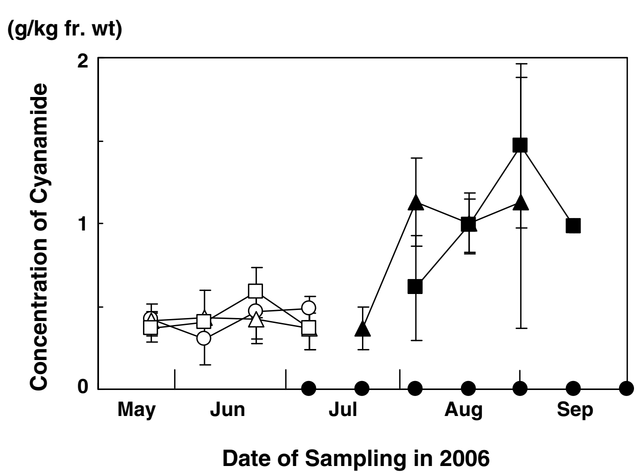

Fig. 1. Cyanamide concentration of *Vicia* species grown in fields. Aerial part of *V. villosa* subsp. *varia* (□, △ and ○), *V. cracca* (■ and ▲), and *V. amoena* (●). Values are mean ($n = 3 \pm$ s.d.).

Table 1
Germination and growth rates of three *Vicia* species in an illuminated chamber

	V. villosa subsp. *varia*	*V. cracca*	*V. amoena*
Weight of 1000 seeds (g)	45.9	13.1	22.8
Germination ratio[A]			
No treatment	49/50	10/50	3/50
Wounding treatment	NT[C]	39/50	32/50
H_2SO_4 treatment	NT[C]	49/50	38/50
Fresh weight of a 4-week-old seedling (mg)[B]	509 ± 58^a	106 ± 21^b	110 ± 16^b

[A] The numbers of germinating seeds were counted 14 d after sowing. Values are shown as the number of germinating seeds per the number of tested seeds.
[B] Values are mean ± s.d. ($n = 10$). Data topped by the same letter do not differ significantly (Fisher's LSD test of ANOVA, $P < 0.05$).
[C] Not tested.

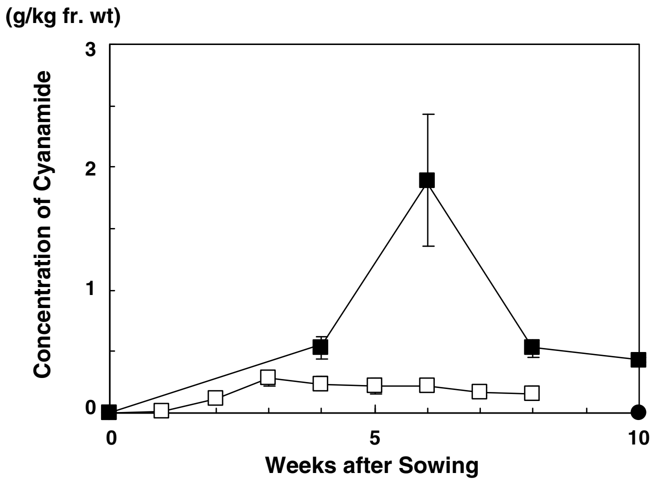

Fig. 2. Cyanamide concentration of *Vicia* species grown in an illuminated chamber. Whole plant of *V. villosa* subsp. *varia* (□), *V. cracca* (■), and *V. amoena* (●). Values are mean ± s.d. (*V. villosa* subsp. *varia*: $n = 5$, *V. cracca* and *V. amoena*: $n = 3$).

germination (Fig. 2). *Vicia cracca* accumulated 1893 ± 539 mg/kg fr. wt after 6 weeks, while *V. villosa* subsp. *varia* 225 ± 43.8 mg/kg fr. wt. Significant differences between the contents in both species after 4 weeks ($F_{1,6} = 28.56$, $P = 0.001754$), 6 weeks ($F_{1,6} = 53.01$, $P = 0.000342$), and 8 weeks ($F_{1,4} = 52.78$, $P = 0.001906$) indicate a lower ability of *V. villosa* subsp. *varia* to accumulate cyanamide than *V. cracca* at least under the tested conditions. Analysis of the cyanamide content in *V. amoena* was conducted only once after 10 weeks due to the lowest growth rate among the three, and no cyanamide in the seedlings was detected. Although another growth condition could give a different result, there is a good correspondence between the levels of the cyanamide contents in these species in the illuminated chamber experiment and those in the field samples.

The localization of cyanamide in the leaves of *V. villosa* subsp. *varia* seedlings grown in the illuminated chamber was also clarified (Table 2). Since this experiment was conducted independently of the comparison of three *Vicia* species shown in Table 1, the total fresh weights do not necessarily correspond to each other due to a slight conditional difference. We measured the cyanamide concentrations in the leaves, stems, and roots, respectively. The highest concentration was observed in the leaves, at least 35 times larger than in the roots. Considering the fresh weight of each part, more than 95% of the total cyanamide in a seedling was present in the leaves plus the stems. Having demonstrated the localization of natural cyanamide in the aerial part would contribute to a basic understanding of its physiological and ecological roles. These still remain to be studied.

Table 2
Contents of cyanamide in a 4-week-old seedling of *V. villosa* subsp. *varia* grown in an illuminated chamber

	Leaf	Stem	Root
Fresh weight (mg/seedling)[a]	101 ± 19.6	117 ± 21.9	131 ± 23.9
Concentration of cyanamide (mg/kg fr. wt)[a,b]	629 ± 209	256 ± 134	<20
Content of cyanamide (μg/seedling)[a]	65.2 ± 30.6	30.6 ± 18.2	Not measured

[a] Five seedlings were used for the experiment. Values are mean ± s.d. ($n = 5$).
[b] Data for the leaf and the stem were significantly distinguishable ($F_{1,8} = 11.3$, $P = 0.009906$).

We also conducted an extensive survey of other higher plants to clarify the distribution of natural cyanamide. Wide varieties of plants including tropical species were collected from the Tsukuba Botanical Garden, which belongs to the National Science Museum of Japan. The leaves of 452 species (131 families, 340 genera) were extracted and analyzed by the SID-GC–MS method. In addition to the six *Vicia* species tested previously (Kamo et al., 2006b), we analyzed the cyanamide contents in *V. amurensis*, *V. japonica*, and *V. pseudo-orobus* in the present study, finding that these three *Vicia* species contained no detectable cyanamide (<1 mg/kg fr. wt). The ability to biosynthesize cyanamide was limited to *V. villosa* subsp. *varia* and *V. cracca* in this genus as far as we have investigated. Although all of the other species in the higher plants tested here also contained no detectable cyanamide, we found a remarkable exception: a leguminous tree, *Robinia pseudo-acacia* L., containing 46 ± 7 mg/kg fr. wt of cyanamide in the leaves. To ensure its occurrence, another leaf sample collected from a young wild-grown tree of this species growing in a different place (Nagano, Japan) was analyzed. It also contained a much larger amount of cyanamide, 442 mg/kg fr. wt, than the sample collected from the botanical garden. Despite the varied contents between the samples from the two individual trees, the occurrence of cyanamide in *R. pseudo-acacia* was confirmed. The leaves of the other *Robinia* species collected from the botanical garden, *R. luxurians*, contained no detectable cyanamide. Among the 553 species we have investigated to date, only three species, *V. villosa* subsp. *varia*, *V. cracca* and *R. pseudo-acacia*, are able to biosynthesize cyanamide.

The distribution of natural cyanamide is limited in a few species in particular genera, and the genera, *Vicia* and *Robinia*, bear weak similarity in morphology. It is possible that a parallel evolution has resulted in such a scattering of cyanamide-biosynthesizing distribution. According to a phylogenetic classification study on leguminous plants, however, the two genera have been categorized in a group together with *Lotus*, *Medicago*, and others (Doyle et al., 1997). This suggests an identical origin of the ability to biosynthesize cyanamide in the evolution process: while the majority of the genera and species in the group, including *V. amoena* and *R. luxurians*, may have lost the ability to biosynthesize cyanamide due to loss of a gene(s) or its expression, *V. villosa* subsp. *varia*, *V. cracca* and *R. pseudo-acacia* may have continued biosynthesizing cyanamide.

3. Conclusions

In conclusion, *Vicia cracca* accumulates larger amounts of cyanamide than *V. villosa* subsp. *varia*, and *V. amoena* has no detectable cyanamide. The leaves rather than the stems or the roots of *V. villosa* subsp. *varia* seedlings contained the majority of cyanamide. The distribution of cyanamide, among the 553 species of higher plants we have investigated so far, is limited to three leguminous plants, *V. villosa* subsp. *varia*, *V. cracca*, and *R. pseudo-acacia*.

4. Experimental

4.1. General experimental procedures

[$^{15}N_2$]Cyanamide (min 99.1 at% ^{15}N, in 51.5% aq. soln.) was purchased from Isotec (Miamisburg, OH, USA).

4.2. Plant materials of Vicia species and Robinia pseudo-acacia

Aerial parts of *V. villosa* subsp. *varia*, *V. cracca*, and *V. amoena* were sampled in Nagano, Japan, between May and September, 2006. Voucher specimens (KPM-NA0127954 for *V. villosa* subsp. *varia*, KPM-NA0127953 for *V. cracca*, and KPM-NA0127990 for *V. amoena*) are deposited at the Kanagawa Prefectural Museum of Natural History, Kanagawa, Japan. Seeds of *V. villosa* subsp. *varia* were purchased from Takii Co., Ltd. (commercially available as "hairy vetch"; Kyoto, Japan), which we identified as not *V. villosa* but *V. villosa* subsp. *varia* judging from the smooth stems of the grown plants. Seeds of *V. cracca* and *V. amoena* were collected in Nagano, Japan, in September and October, 2005. For wounding treatments, seeds of *V. cracca* and *V. amoena* were gently rubbed with a file. For sulfuric acid treatment, they were soaked in conc. H_2SO_4 for 15 min and then washed twice with H_2O. Five seeds were planted in each pot (6 cm diameter × 5.5 cm high) containing vermiculite and incubated in an illuminated growth chamber (LIB-301; Iwaki, Tokyo, Japan) without the application of fertilizer under an illumination cycle of light (16 h) and dark (8 h) at 20 °C. The leaves of *R. pseudo-acacia* were sampled in Nagano, Japan, in August, 2005.

4.3. Plant material collected in a botanical garden

The leaves of the plants described below were collected in the Tsukuba Botanical Garden, which belongs to the National Science Museum of Japan, Ibaraki, Japan, in September, 2005 and May, 2006 (Konishi, 2003): *Acanthus mollis*, *Aphelandra squarrosa* (Acanthaceae), *Acer distylum* f. *dissectum*, *A. mono*, *A. nikoense*, *A. palmatum* var. *palmatum* (Aceraceae), *Cordyline australis* (Agavaceae), *Alisma canaliculatum*, *Echinodorus grandiflorus* subsp. *aureus* (Alismataceae), *Eucharis grandiflora*, *Lycoris sanguinea* (Amaryllidaceae), *Cotinus coggygria*, *Pistacia chinensis* (Anacardiaceae), *Annona cherimola*, *Asimina triloba* (Annonaceae), *Strophanthus divaricatus* (Apocynaceae), *Ilex crenata* var. *paludosa*, *I. crenata*, *I. macropoda*, *I. paraguayensis*, *I. serrata* (Aquifoliaceae), *Acorus gramineus*, *Arisaema limbatum*, *A. sikokianum*, *Lysichiton camtschatcense*, *Monstera deliciosa*, *Spathiphyllum* sp., *Symplocarpus nipponicus* (Araceae), *Acanthopanax sciadophylloides*, *Dendropanax trifidus*, *Evodiopanax innovans*, *Fatsia japonica* (Araliaceae), *Araucaria columnalis* (Araucariaceae) *Cynanchum japonicum*, *Metaplexis japonica* (Asclepiadaceae), *Epimedium grandiflorum* var. *thunbergianum*, *Nandina domestica* (Berberidaceae), *Alnus japonica*, *A. pendula*, *A. trabeculosa*, *Carpinus tschonoskii*, *Corylus sieboldiana*, *Ostrya japonica* (Betulaceae), *Catalpa ovata*, *Jacaranda mimosifolia* (Bignoniaceae), *Blechnum gibbum*, *Struthiopteris niponica*, *Woodwardia orientalis* (Blechnaceae), *Durio zibethinus* (Bombacaceae), *Echium fastuosum*, *E. vulgare*, *Ehretia dicksonii* var. *japonica*, *Symphytum asperum* (Boraginaceae), *Ananas comosus*, *Vriesea splendens* (Bromeliaceae), *Buddleja davidii* (Buddlejaceae), *Bursera fagaroides* (Burseraceae), *Pachysandra terminalis*, *Simmondsia chinensis* (Buxaceae), *Rhodocactus panamensis* (Cactaceae), *Calycanthus floridus* (Calycanthaceae), *Adenophora remotiflora*, *A. triphylla* var. *japonica*, *Campanula punctata* var. *hondoensis*, *Codonopsis ussuriensis* (Campanulaceae), *Abelia spathulata*, *Lonicera gracilipes* var. *glandulosa*, *L. morrowii*, *Viburnum furcatum*, *V. opulus* f. *hydrangeoides*, *V. plicatum* var. *tomentosum*, *V. sieboldii*, *Weigela coraeensis* var. *coraeensis*, *W. floribunda*, *W. hortensis* (Caprifoliaceae), *Dianthus japonicus* (Caryophyllaceae), *Euonymus alatus*, *E. japonicus* var. *japonicus*, *E. oxyphyllus*, *E. sieboldianus* var. *sieboldianus* (Celastraceae), *Cercidiphyllum japonicum* (Cercidiphyllaceae), *Clethra barbinervis* (Clethraceae), *Achillea millefolium*, *Ainsliaea cordifolia* var. *maruoi*, *Artemisia capillaris*, *Aster scaber*, *Atractylodes japonica*, *Chrysanthemum boreale*, *C. japonense* var. *ashizuriense*, *C. makinoi*, *C. pacificum*, *C. wakasaense*, *C. yezoense*, *C. yoshinaganthum*, *Cirsium japonicum*, *C. maritimum*, *Crepidiastrum platyphyllum*, *Cynara cardunculus*, *Eupatorium lindleyanum*, *E. lindleyanum* var. *yasushii*, *Farfugium japonicum* var. *japonicum*, *Helichrysum angustifolium*, *Inula helenium*, *Leontopodium discolor*, *Ligularia hodgsonii*, *Petasites japonicus* var. *giganteus*, *Santolina virens, Senecio cannabifolius, Solidago virgaurea* var. *praeflorens, Syneilesis palmate, Tanacetum vulgare, Taraxacum hondoense, T. platycarpum, Tetragonotheca helianthoides* (Compositae), *Calystegia soldanella* (Convolvulaceae), *Aucuba japonica* var. *japonica*, *Cornus officinalis*, *Helwingia japonica* (Cornaceae), *Sedum kamtschaticum*, *S. lineare*, *S. oryzifolium* (Crassulaceae), *Juniperus chinensis* var. *procumbens*, *J. conferta*, *Thujopsis dolabrata* var. *dolabrata* (Cupressaceae), *Zamia furfuracea* (Cycadaceae), *Carludovica palmata* (Cyclanthaceae), *Carex hachijoensis*, *C. morrowii*, *C. oshimensis*, *C. podogyna*, *Eleocharis parvinux, Machaerina nipponensis, Scirpus juncoides, S. triqueter*

(Cyperaceae) *Daphniphyllum macropodum* var. *humile* (Daphniphyllaceae), *Nephrolepis exaltata 'Marshallii'* (Davalliaceae), *Davidia involucrata* (Davidiaceae), *Dipterocarpus grandiflorus* (Dipterocarpaceae), *Arachniodes standishii*, *Athyriorumohra maximowiczii*, *Athyrium vidalii*, *Christella acuminata*, *C. parasitica*, *Cornopteris decurrenti-alata*, *Cyrtomium falcatum* subsp. *falcatum*, *Deparia japonica*, *D. lancea*, *Dryopsis maximowicziana*, *D. hikonensis*, *D. tokyoensis*, *Matteuccia orientalis*, *Onoclea sensibilis* var. *interrupta*, *Parathelypteris beddomei*, *Polystichum makinoi*, *Thelypteris palustris* (Dryopteridaceae), *Diospyros japonica* (Ebenaceae), *Elaeagnus pungens*, *E. umbellata* var. *umbellata* (Elaeagnaceae), *Ephedra sinica* (Ephedraceae), *Equisetum hyemale* subsp. *hyemale* (Equisetaceae), *Enkianthus campanulatus* var. *sikokianus*, *E. perulatus*, *Pieris japonica*, *Rhododendron amagianum*, *R. kiusianum*, *R. macrosepalum*, *R. quinquefolium*, *R. reticulatum*, *R. tosaense, R. weyrichii* (Ericaceae), *Antidesma venosum*, *Breynia disticha*, *Euphorbia pekinensis* f. *maritima*, *Manihot esculenta* (Euphorbiaceae), *Euptelea polyandra* (Eupteleaceae), *Castanopsis cuspidata* var. *sieboldii*, *Fagus crenata*, *F. japonica*, *Quercus glauca*, *Q. mongolica* var. *grosseserrata* (Fagaceae), *Gentiana triflora* var. *japonica* (Gentianaceae), *Geranium soboliferum* (Geraniaceae), *Aeschynanthus tricolor*, *Nemathanthus gregarious* (Gesneriaceae), *Arundo donax* var. *versicolor*, *Coix lacryma-jobi*, *Cortaderia selloana*, *Cymbopogon citratus*, *Eleusine indica*, *Elymus mollis*, *Festuca ovina*, *Ischaemum aristatum* var. *glaucum*, *Sasa veitchii* (Gramineae), *Clusia cruiva*, *Garcinia subelliptica*, *Hypericum ascyron*, *H. patulum* (Guttiferae), *Hamamelis japonica*, *Loropetalum chinense* (Hamamelidaceae), *Aesculus hippocastanum*, *A. turbinate* (Hippocastanaceae), *Ottelia alismoides* (Hydrocharitaceae), *Curculigo capitulata* (Hypoxidaceae), *Illicium religiosum* (Illiciaceae), *Gladiolus alatus*, *Iris japonica*, *I. pseudacorus*, *I. sanguinea* (Iridaceae), *Agastache rugosa*, *Dracocephalum argunense* var. *japonica*, *Dysophylla yatabeana*, *Iboza riparia*, *Keiskea japonica*, *Lavandula angustifolia*, *Lycopus maackianus*, *Mentha suaveolens*, *Origanum laevigatum 'Herrenhausen'*, *Perovskia atriplicifolia*, *Phlomis fruticosa*, *Plectranthus umbrosus*, *Rosmarinus officinalis*, *Salvia nipponica*, *S. sclarea*, *Satureja hortensis*, *Scutellaria indica*, *S. strigillosa*, *Teucrium massiliense*, *Thymus vulgaris 'Reiter'*, *T. quinquecostatus* (Labiatae), *Cinnamomum zeylanicum*, *Laurus nobilis*, *Lindera glauca*, *Parabenzoin praecox* (Lauraceae), *Napoleona vogelii* (Lecythidaceae), *Acacia karroo*, *Albizia julibrissin*, *A.* sp., *Baptisia alba* var. *macrophylla*, *B. tinctoria*, *Bauhinia japonica*, *Caesalpinia decapetala* var. *japonica*, *C. pulcherrima*, *Cassia nomame*, *C. surattensis*, *Castanospermum australe*, *Cercis chinensis*, *Crotalaria sessiliflora*, *Erythrophleum guineense*, *Genista tinctoria*, *Hedysarum vicioides*, *Laburnum anagyroides*, *Lathyrus japonicus*, *L. quinquenervius*, *Lespedeza bicolor* f. *acutifolia*, *L. cuneata*, *L. cyrtobotrya*, *L. virgata*, *Lotus corniculatus* var. *japonicus*, *Maniltoa browneoides*, *Milletia taiwaniana*, *Pterocarpus indicus*, *Robinia luxurians*, *R. pseudo-acacia*, *Sophora flavescens* var. *angustifolia*, *Spartium junceum*, *Strongylodon macrobotrys*, *Thermopsis lupinoides*, *Trifolium lupinaster*, *Vicia amurensis*, *V. japonica*, *V. pseudo-orobus*, *Wisteria floribunda* (Leguminosae), *Asparagus cochinchinensis* var. *cochinchinensis*, *Convallaria keiskei*, *Disporum pullum*, *D. smilacinum*, *Hemerocallis dumortieri* var. *dumortieri*, *Hosta kikutii*, *H. longissima* var. *brevifolia*, *H. Montana*, *Lilium japonicum*, *L. leichtlinii* var. *tigrinum*, *L. maculatum*, *Liriope platyphylla*, *Ophiopogon jaburan*, *O. planiscapus*, *Ruscus aculeatus*, *Smilax china*, *Tulbaghia violacea*, *Veratrum maackii* var. *parviflorum* (Liliaceae), *Lawsonia inermis* (Lythraceae), *Liriodendron tulipifera*, *Magnolia grandiflora*, *M. kobus* var. *kobus*, *M. obovata*, *M. sieboldii*, *M. stellata* var. *stellata*, *Michelia alba*, *M. figo* (Magnoliaceae), *Malpighia glabra* (Malpighiaceae), *Alcea rosea*, *Hibiscus arnottianus*, *H. syriacus*, *Pavonia intermedia* var. *kermesina* (Malvaceae), *Angiopteris evecta* (Marattiaceae), *Cocculus orbiculatus* (Menispermaceae), *Menyanthes trifoliata* (Menyanthaceae), *Artocarpus heterophyllus*, *Ficus pumila*, *Morus bombycis* var. *maritima* (Moraceae), *Musa acuminata*, *M. liukiuensis* (Musaceae), *Ardisia crenata* (Myrsinaceae), *Psidium guajava* (Myrtaceae), *Bougainvillea spectabilis* (Nyctaginaceae), *Nuphar japonicum* (Nymphaeaceae), *Ochna serrulata* (Ochnaceae), *Chionanthus retusus*, *Forsythia viridissima* var. *viridissima*, *Ligustrum japonicum*, *L. obtusifolium*, *Osmanthus heterophyllus*, *Syringa reticulata*, *S. vulgaris* (Oleaceae), *Botrypus virginianus*, *Sceptridium japonicum* var. *japonicum* (Ophioglossaceae), *Calanthe discolor*, *Epipactis thunbergii*, *Sobralia xantholeuca* (Orchidaceae), *Osmunda lancea* (Osmundaceae), *Paeonia lactiflora* (Paeoniaceae) *Ceratopteris thalictroides* (Parkeriaceae), *Passiflora edulis* (Passifloraceae), *Larix kaempferi*, *Picea abies*, *Picea jezoensis* var. *hondoensis* (Pinaceae), *Piper magnificum*, *P. nigrum* (Piperaceae), *Pittosporum tobira* (Pittosporaceae), *Plagiogyria euphlebia* (Plagiogyriaceae), *Plumbago auriculata* (Plumbaginaceae), *Podocarpus macrophyllus* (Podocarpaceae), *Homalocladium platycladum*, *Neocheiropteris ensata* f. *platyphylla*, *Polygonum cuspidatum* f. *colorans*, *P. suffultum* (Polypodiaceae), *Eichhornia crassipes* (Pontederiaceae), *Lysimachia clethroides*, *L. punctata*, *L. thyrsantha*, *L. vulgaris* var. *davurica*, *Primula japonica* (Primulaceae), *Macadamia integrifolia* (Proteaceae), *Adiantum anceps*, *A. pedatum*, *Coniogramme japonica* subsp. *japonica*, *Dennstaedtia wilfordii*, *Hypolepis punctata*, *Microlepia pseudo-strigosa*, *Onychium japonicum*, *Pteris cretica* (Pteridaceae), *Anemone hepatica* var. *japonica*, *Aquilegia buergeriana* var. *buergeriana*, *Caltha palustris* var. *enkoso* (Ranunculaceae), *Ziziphus jujuba* var. *inermis* (Rhamnaceae), *Aruncus dioicus* var. *insularis*, *Chaenomeles speciosa*, *Cotoneaster horizontalis*, *Geum aleppicum*, *Photinia glabra*, *Potentilla fruticosa* var. *mandshurica*, *P. kleiniana*, *Prunus incisa* f. *yamadei*, *P. lannesiana* var. *speciosa*, *P. mume*, *P. pendula* f. *pendula*, *Pyrus communis 'La France'*, *Rosa hirtula*, *R. multiflora*, *Rubus buergeri*, *R. crataegifolius*, *R. okinawensis*, *R. trifidus*, *Sorbaria sorbifolia* var. *stellipila*, *Spiraea betulifolia*, *S. japonica*, *S. nervosa*, *Stephanandra incise* (Rosaceae), *Coffea Arabica*, *Galium verum* f.

nikkoense, *Gardenia jasminoides* f. *grandiflora*, *Ixora chinensis* (Rubiaceae), *Citrus hanayu*, *Orixa japonica*, *Phellodendron amurense*, *Poncirus trifoliate*, *Zarthoxylum piperitum*, *Z. schinifolium* (Rutaceae), *Populus balsamifera*, *P. maximowiczii*, *Salix bakko*, *S. integra*, *S. subfragilis* (Salicaceae), *Richadella dulcifica* (Sapotaceae), *Sarracenia leucophylla* (Sarraceniaceae), *Saururus chinensis* (Saururaceae), *Astilbe microphylla*, *Hydrangea macrophylla* var. *acuminata*, *H. macrophylla* f. *normalis*, *H. paniculata* (Saxifragaceae), *Lygodium japonicum* (Schizaeaceae), *Digitalis thapsi*, *Pseudolysimachion schmidtianum* (Scrophulariaceae), *Selaginella uncinata* (Selaginellaceae), *Lycium chinense*, *Physalis alkekengi* var. *franchetii*, *Solandra maxima* (Solanaceae), *Sparganium fallax*, *S. stoloniferum* (Sparganiaceae), *Stachyurus praecox* var. *praecox* (Stachyuraceae), *Theobroma cacao* (Sterculiaceae), *Symplocos chinensis* f. *pilosa* (Symplocaceae), *Cunninghamia lanceolata*, *Sequoia sempervirens* (Taxodiaceae), *Camellia sinensis*, *Eurya emarginata*, *Stewartia monadelpha*, *S. pseudo-camellia* (Theaceae), *Edgeworthia chrysantha* (Thymelaeaceae), *Tilia platyphyllos* (Tiliaceae), *Trochodendron aralioides* (Trochodendraceae), *Typha angustata* (Typhaceae), *Cryptotaenia japonica*, *Foeniculum vulgare*, *Peucedanum japonicum* (Umbelliferae), *Boehmeria holosericea* (Urticaceae), *Patrinia scabiosifolia*, *P. villosa* (Valerianaceae), *Callicarpa dichotoma*, *C. japonica* var. *japonica*, *Clerodendrum quadriloculare*, *Lippia nodifloa*, *Vitex rotundifolia* (Verbenaceae), *Viola raddeana*, *V. verecunda* (Violaceae), *Vitis flexuosa*, *V. thunbergii* var. *izu-insularis* (Vitaceae), *Alpinia zerumbet*, *Curcuma longa*, *Elettaria cardamomum*, *Hedychium coronarium*, *Kaempferia roscoeana* (Zingiberaceae).

4.4. Extraction and purification of cyanamide

Fresh plant materials equivalent to 2.0 g fr. wt were immediately cut into pieces 5 mm in length and ground using a ceramic mortar by adding 20 μl of [$^{15}N_2$]cyanamide aq. soln. (1000 ppm) and 3 ml of Me_2CO. To the filtrate of the extract was added DMSO (10 μl) and concentrated by using a rotary evaporator at 30 °C in vacuo. The concd. soln. was diluted with Me_2CO (500 μl) and applied to a silica gel cartridge column (Bond Elut SI, 500 mg; Varian) which was preconditioned with *n*-hexane and pre-filled with 2 ml of *n*-hexane. The column was eluted with Me_2CO-*n*-hexane (2:8, 3:7, and 4:6; each 3 ml). The last two fractions were combined, concentrated by using a rotary evaporator at 30 °C, and then diluted to 2 ml with Me_2CO. To 1 ml of the soln., 10 μl of *m*-(trifluoromethyl)benzonitrile (1000 ppm in MeOH) was added as an internal standard for correcting errors in the GC–MS analysis. A 2-μl portion of the sample soln. was injected to the GC–MS. To analyze samples containing large amounts of natural cyanamide (*V. villosa* subsp. *varia*, *V. cracca*, and *R. pseudo-acacia*), the ratio of the plant material used for the extraction to the internal standard was reduced to 60 μl:0.3 g fr. wt or 60 μl:0.6 g fr. wt.

4.5. GC–MS analysis

The analytical conditions were as follows: GC–MS instrument, QP-5000 (Shimadzu, Kyoto, Japan); analytical column, CP-Sil 8 CB for amines (0.25 mm I.D., 30 m length, 0.25 μm thickness; GL Sciences, Tokyo, Japan); injector temp., 250 °C; interface temp. (ion source temp.), 250 °C; ionizing voltage, 70 eV (EI–MS); signal sampling rate, 0.2 s; injection mode, split-less mode with 30-s of sampling time; injection volume, 2.0 μl; column temp., 50 °C for the initial 5 min followed by an increase in temp. of 15 °C min^{-1} up to 250 °C and kept at 250 °C for 3 min; carrier gas, He; total flow rate, 50 ml min^{-1}; column flow rate, 1 ml min^{-1}. Cyanamide was detected at R_t 8.4 min. Natural cyanamide and [$^{15}N_2$]cyanamide (internal standard for the SID-GC–MS method) were detected as peak areas on the mass chromatograms of the $[M]^+$ ions (*m*/*z* 42 and 44, respectively) and the isotoepic dilution ratio was used to quantify the natural cyanamide content in the samples. The detailed procedure is described by Hiradate et al. (2005).

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